INSECT p53 TUMOR SUPPRESSOR GENES AND PROTEINS

REFERENCE TO RELATED APPLICATION

5   This application is a continuation-in-part of U.S. application no. 09/268,969, filed March 16, 1999; and of U.S. application no. 60/184,373 of same title, filed February 23, 2000. The entire contents of both prior applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10   The p53 gene is mutated in over 50 different types of human cancers, including familial and spontaneous cancers, and is believed to be the most commonly mutated gene in human cancer (Zambetti and Levine, FASEB (1993) 7:855-865; Hollstein, *et al.*, Nucleic Acids Res. (1994) 22:3551-3555). Greater than 90% of mutations in the p53 gene are missense mutations that alter a single amino acid that inactivates p53 function. Aberrant

15 forms of human p53 are associated with poor prognosis, more aggressive tumors, metastasis, and survival rates of less than 5 years (Koshland, Science (1993) 262:1953).

     The human p53 protein normally functions as a central integrator of signals arising from different forms of cellular stress, including DNA damage, hypoxia, nucleotide deprivation, and oncogene activation (Prives, Cell (1998) 95:5-8). In response to these

20 signals, p53 protein levels are greatly increased with the result that the accumulated p53 activates pathways of cell cycle arrest or apoptosis depending on the nature and strength of these signals. Indeed, multiple lines of experimental evidence have pointed to a key role for p53 as a tumor suppressor (Levine, Cell (1997) 88:323-331). For example, homozygous p53 "knockout" mice are developmentally normal but exhibit nearly 100% incidence of

25 neoplasia in the first year of life (Donehower *et al.*, Nature (1992) 356:215-221). The biochemical mechanisms and pathways through which p53 functions in normal and cancerous cells are not fully understood, but one clearly important aspect of p53 function is its activity as a gene-specific transcriptional activator. Among the genes with known p53-response elements are several with well-characterized roles in either regulation of the cell

30 cycle or apoptosis, including GADD45 , p21/Waf1/Cip1, cyclin G, Bax, IGF-BP3, and MDM2 (Levine, Cell (1997) 88:323-331).

Human p53 is a 393 amino acid phosphoprotein which is divided structurally and functionally into distinct domains joined in the following order from N-terminus to C-terminus of the polypeptide chain: (a) a transcriptional activation domain; (b) a sequence-specific DNA-binding domain; (c) a linker domain; (d) an oligomerization domain; and (e) a basic regulatory domain. Other structural details of the p53 protein are in keeping with its function as a sequence-specific gene activator that responds to a variety of stress signals. For example, the most N-terminal domain of p53 is rich in acidic residues, consistent with structural features of other transcriptional activators (Fields and Jang, Science (1990) 249:1046-49). By contrast, the most C-terminal domain of p53 is rich in basic residues, and has the ability to bind single-stranded DNA, double-stranded DNA ends, and internal deletions loops (Jayaraman and Prives, Cell (1995) 81: 1021-1029). The association of the p53 C-terminal basic regulatory domain with these forms of DNA that are generated during DNA repair may trigger conversion of p53 from a latent to an activated state capable of site-specific DNA binding to target genes (Hupp and Lane, Curr. Biol. (1994) 4: 865-875), thereby providing one mechanism to regulate p53 function in response to DNA damage. Importantly, both the N-terminal activation domain and the C-terminal basic regulatory domain of p53 are subject to numerous covalent modifications which correlate with stress-induced signals (Prives, Cell (1998) 95:5-8). For example, the N-terminal activation domain contains residues that are targets for phosphorylation by the DNA-activated protein kinase, the ATM kinase, and the cyclin activated kinase complex. The C-terminal basic regulatory domain contains residues that are targets for phosphorylation by protein kinase-C, cyclin dependent kinase, and casein kinase II, as well as residues that are targets for acetylation by PCAF and p300 acetyl transferases. p53 activity is also modulated by specific non-covalent protein-protein interactions (Ko and Prives, Genes Dev. (1996) 10: 1054-1072). Most notably, the MDM2 protein binds a short, highly conserved protein sequence motif, residues 13-29, in the N-terminal activation domain of p53 (Kussie *et al.*, Science (1996) 274:948-953. As a result of binding p53, MDM2 both represses p53 transcriptional activity and promotes the degradation of p53.

Although several mammalian and vertebrate homologs of the tumor suppressor p53 have been described, only two invertebrate homologs have been identified to date in mollusc and squid. Few lines of evidence, however, have hinted at the existence of a p53 homolog in any other invertebrate species, such as the fruit fly *Drosophila*. Indeed, numerous direct attempts to isolate a *Drosophila* p53 gene by either cross-hybridization or PCR have failed to identify a p53-like gene in this species (Soussi *et al.*, Oncogene (1990)

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5: 945-952). However, other studies of response to DNA damage in insect cells using nucleic cross-hybridization and antibody cross-reactivity have provided suggestive evidence for existence of p53-, p21-, and MDM2-like genes (Bae *et al.*, Exp Cell Res (1995) 375:105-106; Yakes, 1994, Ph.D. thesis, Wayne State University). Nonetheless, no isolated insect p53 genes or proteins have been reported to date.

Identification of novel p53 orthologues in model organisms such as *Drosophila melanogaster* and other insect species provides important and useful tools for genetic and molecular study and validation of these molecules as potential pharmaceutical and pesticide targets. The present invention discloses insect p53 genes and proteins from a variety of diverse insect species. In addition, *Drosophila* homologs of p33 and Rb genes, which are also involved in tumor suppression, are described.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide insect p53 nucleic acid and protein sequences that can be used in genetic screening methods to characterize pathways that p53 may be involved in as well as other interacting genetic pathways. It is also an object of the invention to provide methods for screening compounds that interact with p53 such as those that may have utility as therapeutics.

These and other objects are provided by the present invention which concerns the identification and characterization of insect p53 genes and proteins in a variety of insect species. Isolated nucleic acid molecules are provided that comprise nucleic acid sequences encoding p53 polypeptides and derivatives thereof. Vectors and host cells comprising the p53 nucleic acid molecules are also described, as well as metazoan invertebrate organisms (*e.g.* insects, coelomates and pseudocoelomates) that are genetically modified to express or mis-express a p53 protein.

An important utility of the insect p53 nucleic acids and proteins is that they can be used in screening assays to identify candidate compounds which are potential therapeutics or pesticides that interact with p53 proteins. Such assays typically comprise contacting a p53 polypeptide with one or more candidate molecules, and detecting any interaction between the candidate compound and the p53 polypeptide. The assays may comprise adding the candidate molecules to cultures of cells genetically engineered to express p53 proteins, or alternatively, administering the candidate compound to a metazoan invertebrate organism genetically engineered to express p53 protein.

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The genetically engineered metazoan invertebrate animals of the invention can also be used in methods for studying p53 activity, or for validating therapeutic or pesticidal strategies based on manipulation of the p53 pathway. These methods typically involve detecting the phenotype caused by the expression or mis-expression of the p53 protein. The

5   methods may additionally comprise observing a second animal that has the same genetic modification as the first animal and, additionally has a mutation in a gene of interest. Any difference between the phenotypes of the two animals identifies the gene of interest as capable of modifying the function of the gene encoding the p53 protein.

10   **BRIEF DESCRIPTION OF THE FIGURE**

Figures 1A-1B show a CLUSTALW alignment of the amino acid sequences of the insect p53 proteins identified from *Drosophila, Leptinotarsa, Tribolium*, and *Heliothis*, with p53 sequences previously identified in human, *Xenopus*, and squid. Identical amino acid residues within the alignment are grouped within solid lines and similar amino acid residues

15   are grouped within dashed lines.

DETAILED DESCRIPTION OF THE INVENTION

The use of invertebrate model organism genetics and related technologies can greatly facilitate the elucidation of biological pathways (Scangos, Nat. Biotechnol. (1997)

20   15:1220-1221; Margolis and Duyk, Nature Biotech. (1998) 16:311). Of particular use is the insect model organism, *Drosophila melanogaster* (hereinafter referred to generally as "*Drosophila*"). An extensive search for p53 nucleic acid and its encoded protein in *Drosophila* was conducted in an attempt to identify new and useful tools for probing the function and regulation of the p53 genes, and for use as targets in drug discovery. p53

25   nucleic acid has also been identified in the following additional insect species: *Leptinotarsa decemilineata* (Colorado potato beetle, hereinafter referred to as *Leptinotarsa*), *Tribolium castaneum* (flour beetle, hereinafter referred to as *Tribolium*), and *Heliothis virescens* (tobacco budworm, hereinafter referred to as *Heliothis*).

The newly identified insect p53 nucleic acids can be used for the generation of

30   mutant phenotypes in animal models or in living cells that can be used to study regulation of p53, and the use of p53 as a drug or pesticide target. Due to the ability to rapidly carry out large-scale, systematic genetic screens, the use of invertebrate model organisms such as *Drosophila* has great utility for analyzing the expression and mis-expression of p53 protein. Thus, the invention provides a superior approach for identifying other components involved

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in the synthesis, activity, and regulation of p53 proteins. Systematic genetic analysis of p53 using invertebrate model organisms can lead to the identification and validation of compound targets directed to components of the p53 pathway. Model organisms or cultured cells that have been genetically engineered to express p53 can be used to screen candidate compounds for their ability to modulate p53 expression or activity, and thus are useful in the identification of new drug targets, therapeutic agents, diagnostics and prognostics useful in the treatment of disorders associated with cell cycle, DNA repair, and apoptosis. The details of the conditions used for the identification and/or isolation of insect p53 nucleic acids and proteins are described in the Examples section below. Various non-limiting embodiments of the invention, applications and uses of the insect p53 genes and proteins are discussed in the following sections. The entire contents of all references, including patent applications, cited herein are incorporated by reference in their entireties for all purposes. Additionally, the citation of a reference in the preceding background section is not an admission of prior art against the claims appended hereto.

p53 Nucleic Acids

The following nucleic acid sequences encoding insect p53 are described herein: SEQ ID NO:1, isolated from *Drosophila*, and referred to herein as DMp53; SEQ ID NO:3, isolated from *Leptinotarsa*, and referred to herein as CPBp53; SEQ ID NO:5 and SEQ ID NO:7, isolated from *Tribolium*, and referred to herein as TRIB-Ap53 and TRIB-Bp53, respectively; and SEQ ID NO:9, isolated from *Heliothis*, and referred to herein as HELIOp53. The genomic sequence of the DMp53 gene is provided in SEQ ID NO:18.

In addition to the fragments and derivatives of SEQ ID NOs:1, 3, 5, 7, 9, and 18, as described in detail below, the invention includes the reverse complements thereof. Also, the subject nucleic acid sequences, derivatives and fragments thereof may be RNA molecules comprising the nucleotide sequences of SEQ ID NOs:1, 3, 5, 7, 9, and 18 (or derivative or fragment thereof) wherein the base U (uracil) is substituted for the base T (thymine). The DNA and RNA sequences of the invention can be single- or double-stranded. Thus, the term "isolated nucleic acid sequence" or "isolated nucleic acid molecule", as used herein, includes the reverse complement, RNA equivalent, DNA or RNA single- or double-stranded sequences, and DNA/RNA hybrids of the sequence being described, unless otherwise indicated.

Fragments of the p53 nucleic acid sequences can be used for a variety of purposes. Interfering RNA (RNAi) fragments, particularly double-stranded (ds) RNAi, can be used to

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generate loss-of-function phenotypes. p53 nucleic acid fragments are also useful as nucleic acid hybridization probes and replication/amplification primers. Certain "antisense" fragments, i.e. that are reverse complements of portions of the coding sequence of any of SEQ ID NO:1, 3, 5, 7, 9, or 18 have utility in inhibiting the function of p53 proteins. The

5 fragments are of length sufficient to specifically hybridize with the corresponding SEQ ID NO:1, 3, 5, 7, 9, or 18. The fragments consist of or comprise at least 12, preferably at least 24, more preferably at least 36, and more preferably at least 96 contiguous nucleotides of any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18. When the fragments are flanked by other nucleic acid sequences, the total length of the combined nucleic acid sequence is less than

10 15 kb, preferably less than 10 kb or less than 5kb, more preferably less than 2 kb, and in some cases, preferably less than 500 bases. Preferred p53 nucleic acid fragments comprise regulatory elements that may reside in the 5' UTR and/or encode one or more of the following domains: an activation domain, a DNA binding domain, a linker domain, an oligomerization domain, and a basic regulatory domain. The approximate locations of these

15 regions in SEQ ID Nos 1, 3, and 5, and in the corresponding amino acid sequences of SEQ ID Nos 2, 4, and 6, 8, are provided in Table 1.

TABLE 1

| | SEQ ID NOs | | |
	1/2	3/4	5/6
Insect Genus	*Drosophila*	*Leptinotarsa*	*Tribolium*
5' UTR	na 1-111	na 1-120	na 1-93
Activation Domain	na 112-257 aa 1-48	na 121-300 aa 1-60	na 94-277 aa 1-60
DNA Binding Domain	na 366-954 aa 85-280	na 321-936 aa 67-271	na 280-892 aa 62-265
Linker Domain	na 999-1056 aa 296-314	na 937-999 aa 272-292	na 893-958 aa 266-287
Oligomerization Domain	na 1065-1170 aa 318-352	na 1000-1113 aa 293-330	na 959-1075 aa 288-326
Basic Regulatory Domain	na 1179-1269 aa 356-385	na 1114-1182 aa 331-353	na 1076-1147 aa 327-350

20 Further preferred are fragments of bases 354-495 of SEQ ID NO:7 and bases 315-414 of SEQ ID NO:9 of at least 12, preferably at least 24, more preferably at least 36, and most preferably at least 96 contiguous nucleotides.

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The subject nucleic acid sequences may consist solely of any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or fragments thereof. Alternatively, the subject nucleic acid sequences and fragments thereof may be joined to other components such as labels, peptides, agents that facilitate transport across cell membranes, hybridization-triggered cleavage agents or intercalating agents. The subject nucleic acid sequences and fragments thereof may also be joined to other nucleic acid sequences (i.e. they may comprise part of larger sequences) and are of synthetic/non-natural sequences and/or are isolated and/or are purified, i.e. unaccompanied by at least some of the material with which it is associated in its natural state. Preferably, the isolated nucleic acids constitute at least about 0.5%, and more preferably at least about 5% by weight of the total nucleic acid present in a given fraction, and are preferably recombinant, meaning that they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome.

Derivative nucleic acid sequences of p53 include sequences that hybridize to the nucleic acid sequence of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringency conditions such that the hybridizing derivative nucleic acid is related to the subject nucleic acid by a certain degree of sequence identity. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule. Stringency of hybridization refers to conditions under which nucleic acids are hybridizable. The degree of stringency can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. As used herein, the term "stringent hybridization conditions" are those normally used by one of skill in the art to establish at least about a 90% sequence identity between complementary pieces of DNA or DNA and RNA. "Moderately stringent hybridization conditions" are used to find derivatives having at least about a 70% sequence identity. Finally, "low-stringency hybridization conditions" are used to isolate derivative nucleic acid molecules that share at least about 50% sequence identity with the subject nucleic acid sequence.

The ultimate hybridization stringency reflects both the actual hybridization conditions as well as the washing conditions following the hybridization, and it is well known in the art how to vary the conditions to obtain the desired result. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). A preferred derivative nucleic acid is

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capable of hybridizing to any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18 under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05%

5   sodium pyrophosphate and 100 μg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1 h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

Derivative nucleic acid sequences that have at least about 70% sequence identity
10   with any one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 are capable of hybridizing to any one of SEQ ID NO:1, 3, 5, 7, 9, and 18 under moderately stringent conditions that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20 h at 40° C in a
15   solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Other preferred derivative nucleic acid sequences are capable of hybridizing to any
20   one of SEQ ID NOs:1, 3, 5, 7, 9, and 18 under low stringency conditions that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

25   As used herein, "percent (%) nucleic acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides in the candidate derivative nucleic acid sequence identical with the nucleotides in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence
30   identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410; http://blast.wustl.edu/blast/README.html; hereinafter referred to generally as "BLAST") with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database

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against which the sequence of interest is being searched. A percent (%) nucleic acid sequence identity value is determined by the number of matching identical nucleotides divided by the sequence length for which the percent identity is being reported.

Derivative p53 nucleic acid sequences usually have at least 50% sequence identity, preferably at least 60%, 70%, or 80% sequence identity, more preferably at least 85% sequence identity, still more preferably at least 90% sequence identity, and most preferably at least 95% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, or domain-encoding regions thereof.

In one preferred embodiment, the derivative nucleic acid encodes a polypeptide comprising a p53 amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, or 10, or a fragment or derivative thereof as described further below under the subheading "p53 proteins". A derivative p53 nucleic acid sequence, or fragment thereof, may comprise 100% sequence identity with any one of SEQ ID NOs:1, 3, 5, 7, 9, or 18, but be a derivative thereof in the sense that it has one or more modifications at the base or sugar moiety, or phosphate backbone. Examples of modifications are well known in the art (Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such derivatives may be used to provide modified stability or any other desired property.

Another type of derivative of the subject nucleic acid sequences includes corresponding humanized sequences. A humanized nucleic acid sequence is one in which one or more codons has been substituted with a codon that is more commonly used in human genes. Preferably, a sufficient number of codons have been substituted such that a higher level expression is achieved in mammalian cells than what would otherwise be achieved without the substitutions. The following list shows, for each amino acid, the calculated codon frequency (number in parentheses) in humans genes for 1000 codons (Wada *et al.*, Nucleic Acids Research (1990) 18(Suppl.):2367-2411):

Human codon frequency per 1000 codons:

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ARG:  CGA (5.4), CGC (11.3), CGG (10.4), CGU (4.7), AGA (9.9), AGG (11.1)
LEU:  CUA (6.2), CUC (19.9), CUG (42.5), CUU (10.7), UUA (5.3), UUG (11.0)
SER:  UCA (9.3), UCC (17.7), UCG (4.2), UCU (13.2), AGC (18.7), AGU (9.4)
THR:  ACA (14.4), ACC (23.0), ACG (6.7), ACU (12.7)
PRO:  CCA (14.6), CCC (20.0), CCG (6.6), CCU (15.5)
ALA:  GCA (14.0), GCC (29.1), GCG (7.2), GCU (19.6)
GLY:  GGA (17.1), GGC (25.4), GGG (17.3), GGU (11.2)
VAL:  GUA (5.9), GUC (16.3), GUG (30.9), GUU (10.4)
LYS:  AAA (22.2), AAG (34.9)
ASN:  AAC (22.6), AAU (16.6)
GLN:  CAA (11.1), CAG (33.6)
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HIS:    CAC (14.2), CAU (9.3)
GLU:    GAA (26.8), GAG (41.4)
ASP:    GAC (29.0), GAU (21.7)
TYR:    UAC (18.8), UAU (12.5)
CYS:    UGC (14.5), UGU (9.9)
PHE:    UUU (22.6), UUC (15.8)
ILE:    AUA (5.8), AUC (24.3), AUU (14.9)
MET:    AUG (22.3)
TRP:    UGG (13.8)
TER:    UAA (0.7), AUG (0.5), UGA (1.2)
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Thus, a p53 nucleic acid sequence in which the glutamic acid codon, GAA has been replaced with the codon GAG, which is more commonly used in human genes, is an example of a humanized p53 nucleic acid sequence. A detailed discussion of the humanization of nucleic acid sequences is provided in U.S. Pat. No. 5,874,304 to Zolotukhin *et al.* Similarly, other nucleic acid derivatives can be generated with codon usage optimized for expression in other organisms, such as yeasts, bacteria, and plants, where it is desired to engineer the expression of p53 proteins by using specific codons chosen according to the preferred codons used in highly expressed genes in each organism. More specific embodiments of preferred p53 proteins, fragments, and derivatives are discussed further below in connection under the subheading "p53 proteins".

Nucleic acid encoding the amino acid sequence of any of SEQ ID NOs:2, 4, 6, 8, and 10, or fragment or derivative thereof, may be obtained from an appropriate cDNA library prepared from any eukaryotic species that encodes p53 proteins such as vertebrates, preferably mammalian (*e.g.* primate, porcine, bovine, feline, equine, and canine species, *etc.*) and invertebrates, such as arthropods, particularly insects species (preferably *Drosophila, Tribolium, Leptinotarsa, and Heliothis*), acarids, crustacea, molluscs, nematodes, and other worms. An expression library can be constructed using known methods. For example, mRNA can be isolated to make cDNA which is ligated into a suitable expression vector for expression in a host cell into which it is introduced. Various screening assays can then be used to select for the gene or gene product (*e.g.* oligonucleotides of at least about 20 to 80 bases designed to identify the gene of interest, or labeled antibodies that specifically bind to the gene product). The gene and/or gene product can then be recovered from the host cell using known techniques.

Polymerase chain reaction (PCR) can also be used to isolate nucleic acids of the p53 genes where oligonucleotide primers representing fragmentary sequences of interest amplify RNA or DNA sequences from a source such as a genomic or cDNA library (as

described by Sambrook *et al.*, *supra*). Additionally, degenerate primers for amplifying homologs from any species of interest may be used. Once a PCR product of appropriate size and sequence is obtained, it may be cloned and sequenced by standard techniques, and utilized as a probe to isolate a complete cDNA or genomic clone.

5   Fragmentary sequences of p53 nucleic acids and derivatives may be synthesized by known methods. For example, oligonucleotides may be synthesized using an automated DNA synthesizer available from commercial suppliers (*e.g.* Biosearch, Novato, CA; Perkin-Elmer Applied Biosystems, Foster City, CA). Antisense RNA sequences can be produced intracellularly by transcription from an exogenous sequence, *e.g.* from vectors that contain

10   antisense p53 nucleic acid sequences. Newly generated sequences may be identified and isolated using standard methods.

An isolated p53 nucleic acid sequence can be inserted into any appropriate cloning vector, for example bacteriophages such as lambda derivatives, or plasmids such as PBR322, pUC plasmid derivatives and the Bluescript vector (Stratagene, San Diego, CA).

15   Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, *etc.*, or into a transgenic animal such as a fly. The transformed cells can be cultured to generate large quantities of the p53 nucleic acid. Suitable methods for isolating and producing the subject nucleic acid sequences are well-known in the art (Sambrook *et al.*, *supra*; DNA Cloning: A Practical Approach, Vol. 1, 2, 3, 4, (1995)

20   Glover, ed., MRL Press, Ltd., Oxford, U.K.).

The nucleotide sequence encoding a p53 protein or fragment or derivative thereof, can be inserted into any appropriate expression vector for the transcription and translation of the inserted protein-coding sequence. Alternatively, the necessary transcriptional and translational signals can be supplied by the native p53 gene and/or its flanking regions. A

25   variety of host-vector systems may be utilized to express the protein-coding sequence such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. If expression in plants is desired, a variety of transformation constructs, vectors and

30   methods are known in the art (see U.S. Pat. No. 6,002,068 for review). Expression of a p53 protein may be controlled by a suitable promoter/enhancer element. In addition, a host cell strain may be selected which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired

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To detect expression of the p53 gene product, the expression vector can comprise a promoter operably linked to a p53 gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the p53 gene product based on the physical or functional properties of the p53 protein in *in vitro* assay systems (*e.g.* immunoassays or cell cycle assays). The p53 protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product as described above.

Once a recombinant that expresses the p53 gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). The amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant and can thus be synthesized by standard chemical methods (Hunkapiller *et al.*, Nature (1984) 310:105-111). Alternatively, native p53 proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification).

p33 and Rb Nucleic Acids

The invention also provides nucleic acid sequences for *Drosophila* p33 (SEQ ID NO:19), and Rb (SEQ ID NO:21) tumor suppressors. Derivatives and fragments of these sequences can be prepared as described above for the p53 sequences. Preferred fragments and derivatives comprise the same number of contiguous nucleotides or same degrees of percent identity as described above for p53 nucleic acid sequences. The disclosure below regarding various uses of p53 tumor suppressor nucleic acids and proteins (*e.g.* transgenic animals, tumor suppressor assays, *etc.*) also applies to the p33 and Rb tumor suppressor sequences disclosed herein.

p53 Proteins

The CLUSTALW program (Thompson, *et al.*, Nucleic Acids Research (1994) 22(22):4673-4680) was used to align the insect p53 proteins described herein with p53 proteins from human (Zakut-Houri *et al.*, EMBO J. (1985) 4:1251-1255; GenBank gi:129369), *Xenopus* (Sousi *et al.*, Oncogene (1987) 1:71-78; GenBank gi:129374), and squid (GenBank gi:1244762). The alignment generated is shown in Figure 1 and reveals a number of features in the insect p53 proteins that are characteristic of the previously-

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identified p53 proteins. With respect to general areas of structural similarity, the DMp53, CPBp53, and TRIB-Ap53 proteins can be roughly divided into three regions: a central region which exhibits a high degree of sequence homology with other known p53 family proteins and which roughly corresponds to the DNA binding domain of this protein family

5 (Cho *et al.*, Science (1994) 265:346-355), and flanking N-terminal and C-terminal regions which exhibit significantly less homology but which correspond in overall size to other p53 family proteins. The fragmentary polypeptide sequences encoded by the TRIB-Bp53 and HELIOp53 cDNAs are shown by the multiple sequence alignment to be derived from the central region – the conserved DNA-binding domain. Significantly, the protein sequence

10 alignment allowed the assignment of the domains in the DMp53, CPBp53, and TRIB-A p53 proteins listed in Table 1 above, based on sequence homology with previously characterized domains of human p53 (Sousi and May, J. Mol Biol (1996) 260:623-637; Levine, *supra*; Prives, Cell (1998) 95:5-8).

Importantly, the most conserved central regions of the DMp53, CPBp53, and TRIB-

15 A p53 proteins correspond almost precisely to the known functional boundaries of the DNA binding domain of human p53, indicating that these proteins are likely to exhibit similar DNA binding properties to those of human p53. A detailed examination of the conserved residues in this domain further emphasizes the likely structural and functional similarities between human p53 and the insect p53 proteins. First, residues of the human p53 known to

20 be involved in direct DNA contacts (K120, S241, R248, R273, C277, and R280) correspond to identical or similar residues in the DMp53 protein (K113, S230, R234, K259, C263, and R266), and identical residues in the CPBp53 protein (K92, S216, R224, R249, C253, and R256), and the TRIB-Ap53 protein (K88, S213, R220, R245, C249, and R252). Also, with regard to the overall folding of this domain, it was notable that four key residues that

25 coordinate the zinc ligand in the DNA binding domain of human p53 (C176, H179, C238, and C242) are precisely conserved in the DMp53 protein (C156, H159, C227, and C231), the CPBp53 protein (C147, H150, C213, and C217), and the TRIB-A p53 protein (C144, H147, C210, C214). Furthermore, it was striking that the mutational hot spots in human p53 most frequently altered in cancer (R175, G245, R248, R249, R273, and R282), are

30 either identical or conserved amino acid residues in the corresponding positions of the DMp53 protein (R155, G233, R234, K235, K259, and R268), the CPBp53 protein (R146, G221, R224, R225, R249, and K258), and the TRIB-Ap53 protein (R143, G217, R220, R221, R245, and K254).

Interestingly, the insect p53s also have distinct differences from the Human, *Xenopus*, and squid p53s. Specifically, insect p53s contain a unique amino acid sequence within the DNA recognition domain that has the following sequence: (R or K)(I or V)C(S or T)CPKRD. Specifically, amino acid residues 259 to 267 of DMp53 have the sequence:

5 KICTCPKRD; residues 249 to 257 of CPBp53 have the sequence: RICSCPKRD; and residues 245-253 of TRIB-Ap53 have the sequence: RVCSCPKRD. This is in distinct contrast to the Human, *Xenopus*, and squid p53s which have the following corresponding sequence: R(I or V)CACPGRD.

Another region of insect p53s that distinctly differs from previously identified p53s

10 lies in the zinc coordination region of the DNA binding domain. The following sequence is conserved within the insect p53s: FXC(K or Q)NSC (where X = any amino acid). Specifically, residues 225-231 of DMp53 have the sequence: FVCQNSC; residues 211-217 of CPBp53 and residues 208-214 of TRIB-Ap53 have the sequence FVCKNSC; and the corresponding residues in Helio-p53, as shown in Figure 1, have the sequence: FSCKNSC.

15 In contrast, the corresponding sequence in Human and *Xenopus* p53 is YMCNSSC, and in squid it is FMCLGSC.

The high degree of structural homology in the presumptive DNA binding domain of the insect p53 proteins has important implications for engineering derivative (*e.g.* mutant) forms of these p53 genes for tests of function *in vitro* and *in vivo*, and for genetic dissection

20 or manipulation of the p53 pathway in transgenic insects or insect cell lines. Dominant negative forms of human p53 have been generated by creating altered proteins which have a defective DNA binding domain, but which retain a functional oligomerization domain (Brachman *et al.*, Proc Natl Acad Sci USA (1996) 93:4091-4095). Such dominant negative mutant forms are extremely useful for determining the effects of loss-of-function of p53 in

25 assays of interest. Thus, mutations in highly conserved positions within the DNA binding domain of the insect p53 proteins, which correspond to residues known to be important for the structure and function of human p53 (such as R175H, H179N, and R280T of human p53), are likely to result in dominant negative forms of insect p53 proteins. For example, specific mutations in the DMp53 protein to create dominant negative mutant forms of the

30 protein include R155H, H159N, and R266T and for the TRIB-A p53 protein include R143H, H147N, and R252T.

Although other domains of the insect p53 proteins, aside from the DNA binding domain, exhibit significantly less homology compared to the known p53 family proteins, the sequence alignment provides important information about their structure and potential

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function. Notably, just as in the human p53 protein, the C-terminal 20-25 amino acids of the protein comprise a putative region that extends beyond the oligomerization domain, suggesting an analogous function for this region of the insect p53 proteins in regulating activity of the protein. Since deletion of the C-terminal regulatory domain in human p53

5   has been shown to generate constitutively activated forms of the protein (Hupp and Lane, Curr. Biol. (1994) 4:865-875), it is expected that removal of most or all of the corresponding regulatory domain from the insect p53 proteins will generate an activated protein form. Thus preferred truncated forms of the insect p53 proteins lack at least 10 C-terminal amino acids, more preferably at least 15 amino acids, and most preferably at least

10   20 C-terminal amino acids. For example, a preferred truncated version of DMp53 comprises amino acid residues 1-376, more preferably residues 1-371, and most preferably residues 1-366 of SEQ ID NO:2. Such constitutively activated mutant forms of the protein are very useful for tests of protein function using *in vivo* and *in vitro* assays, as well as for genetic analysis.

15   The oligomerization domain of the insect p53 proteins exhibit very limited skeletal sequence homology with other p53 family proteins, although the length of this region is similar to that of other p53 family proteins. The extent of sequence divergence in this region of the insect proteins raises the possibility that the insect p53 protein may be unable to form hetero-oligomers with p53 proteins from vertebrates or squid. And, although the

20   linker domain located between the DNA binding and oligomerization domains also exhibits relatively little sequence conservation, this region of any of the DMp53, CPBp53, and TRIB-A p53 proteins contains predicted nuclear localization signals similar to those identified in human p53 (Shaulsky *et al.*, Mol Cell Biol (1990) 10:6565-6577).

             The activation domain at the N-terminus of the insect p53 proteins also exhibits

25   little sequence identity with other p53 family proteins, although the size of this region is roughly the same as that of human p53. Nonetheless, an important feature of this domain is the relative concentration of acidic residues in the insect p53 proteins. Consequently, it is likely that this N-terminal domain of any of the DMp53, CPBp53, and TRIB-Ap53 proteins will similarly exert the functional activity of a transcriptional activation domain to that of

30   the human p53 domain (Thut *et al.*, Science (1995) 267:100-104). Interestingly, the DMp53, CPBp53 and TRIB-A p53 proteins do not appear to possess a highly conserved sequence motif, FxxLWxxL, found at the N-terminus of vertebrate and squid p53 family proteins. In the human p53 gene, these conserved residues in this motif participate in a

specific interaction between human p53 proteins and mdm2 (Kussie *et al.*, Science (1996) 274:948-953).

It is important to note that, although there is no sequence similarity between the insect p53s and other p53 family members in the C- and N-termini, these regions of p53 contain secondary structure characteristic of p53-related proteins. For example, the human p53 binds DNA as a homo-tetramer and self-association is mediated by a β-sheet and amphipathic α-helix located in the C-terminus of the protein. A similar β-sheet-turn-α-helix is predicted in the C-terminus of DMp53. Further, the N-terminus of the human p53 is a region that includes a transactivation domain and residues critical for binding to the mdm-2 protein. The N-terminus of the DMp53 also include acidic amino acids and likely functions as a transactivation domain.

p53 proteins of the invention comprise or consist of an amino acid sequence of any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments or derivatives thereof. Compositions comprising these proteins may consist essentially of the p53 protein, fragments, or derivatives, or may comprise additional components (*e.g.* pharmaceutically acceptable carriers or excipients, culture media, *etc.*). p53 protein derivatives typically share a certain degree of sequence identity or sequence similarity with any one of SEQ ID NOs:2, 4, 6, 8, and 10 or fragments thereof. As used herein, "percent (%) amino acid sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of amino acids in the candidate derivative amino acid sequence identical with the amino acid in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by BLAST (Altschul *et al.*, *supra*) using the same parameters discussed above for derivative nucleic acid sequences. A % amino acid sequence identity value is determined by the number of matching identical amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation. A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine;

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interchangeable basic amino acids arginine, lysine and histidine; interchangeable acidic amino acids aspartic acid and glutamic acid; and interchangeable small amino acids alanine, serine, cystine, threonine, and glycine.

In one preferred embodiment, a p53 protein derivative shares at least 50% sequence identity or similarity, preferably at least 60%, 70%, or 80% sequence identity or similarity, more preferably at least 85% sequence similarity or identity, still more preferably at least 90% sequence similarity or identity, and most preferably at least 95% sequence identity or similarity with a contiguous stretch of at least 10 amino acids, preferably at least 25 amino acids, more preferably at least 40 amino acids, still more preferably at least 50 amino acids, more preferably at least 100 amino acids, and in some cases, the entire length of any one of SEQ ID NOs:2, 4, 6, 8, or 10. Further preferred derivatives share these % sequence identities with the domains of SEQ ID NOs 2, 4 and 6 listed in Table I above. Additional preferred derivatives comprise a sequence that shares 100% similarity with any contiguous stretch of at least 10 amino acids, preferably at least 12, more preferably at least 15, and most preferably at least 20 amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof. Further preferred fragments comprise at least 7 contiguous amino acids, preferably at least 9, more preferably at least 12, and most preferably at least 17 contiguous amino acids of any of SEQ ID NOs 2, 4, 6, 8, and 10, and preferably functional domains thereof.

Other preferred p53 polypeptides, fragments or derivatives consist of or comprise a sequence selected from the group consisting of RICSCPKRD, KICSCPKRD, RVCSCPKRD, KVCSCPKRD, RICTCPKRD, KICTCPKRD, RVCTCPKRD, and KVCTCPKRD (i.e. sequences of the formula: (R or K)(I or V)C(S or T)CPKRD). Additional preferred p53 polypeptides, fragments or derivatives, consist of or comprise a sequence selected from the group consisting of FXCKNSC and FXCQNSC, where X = any amino acid.

The fragment or derivative of any of the p53 proteins is preferably "functionally active" meaning that the p53 protein derivative or fragment exhibits one or more functional activities associated with a full-length, wild-type p53 protein comprising the amino acid sequence of any of SEQ ID NOs:2, 4, 6, 8, or 10. As one example, a fragment or derivative may have antigenicity such that it can be used in immunoassays, for immunization, for inhibition of p53 activity, *etc*, as discussed further below regarding generation of antibodies to p53 proteins. Preferably, a functionally active p53 fragment or derivative is one that displays one or more biological activities associated with p53 proteins such as regulation of

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the cell cycle, or transcription control. The functional activity of p53 proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey). Example 12 below describes a variety of suitable assays for assessing p53

5   function.

P 53 derivatives can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned p53 gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) (Wells *et al.*, Philos. Trans. R. Soc. London SerA (1986) 317:415),

10   followed by further enzymatic modification if desired, isolated, and ligated *in vitro*, and expressed to produce the desired derivative. Alternatively, a p53 gene can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. A variety of

15   mutagenesis techniques are known in the art such as chemical mutagenesis, *in vitro* site-directed mutagenesis (Carter *et al.*, Nucl. Acids Res. (1986) 13:4331), use of TAB® linkers (available from Pharmacia and Upjohn, Kalamazoo, MI), *etc.*

At the protein level, manipulations include post translational modification, *e.g.* glycosylation, acetylation, phosphorylation, amidation, derivatization by known

20   protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications may be carried out by known technique (*e.g.* specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*). Derivative proteins can also be chemically

25   synthesized by use of a peptide synthesizer, for example to introduce nonclassical amino acids or chemical amino acid analogs as substitutions or additions into the p53 protein sequence.

Chimeric or fusion proteins can be made comprising a p53 protein or fragment thereof (preferably comprising one or more structural or functional domains of the p53

30   protein) joined at its N- or C-terminus via a peptide bond to an amino acid sequence of a different protein. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other in the proper coding frame using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer.

p33 and Rb Proteins

The invention also provides amino acid sequences for *Drosophila* p33 (SEQ ID NO:20), and Rb (SEQ ID NO:22) tumor suppressors. Derivatives and fragments of these

5   sequences can be prepared as described above for the p53 protein sequences. Preferred fragments and derivatives comprise the same number of contiguous amino acids or same degrees of percent identity or similarity as described above for p53 amino acid sequences.

p53 Gene Regulatory Elements

10   p53 gene regulatory DNA elements, such as enhancers or promoters that reside within the 5' UTRs of SEQ ID NOs 1, 3, and 5, as shown in Table I above, or within nucleotides 1-1225 of SEQ ID NO:18, can be used to identify tissues, cells, genes and factors that specifically control p53 protein production. Preferably at least 20, more preferably at least 25, and most preferably at least 50 contiguous nucleotides within the 5'

15   UTRs are used. Analyzing components that are specific to p53 protein function can lead to an understanding of how to manipulate these regulatory processes, for either pesticide or therapeutic applications, as well as an understanding of how to diagnose dysfunction in these processes.

Gene fusions with the p53 regulatory elements can be made. For compact genes that

20   have relatively few and small intervening sequences, such as those described herein for *Drosophila*, it is typically the case that the regulatory elements that control spatial and temporal expression patterns are found in the DNA immediately upstream of the coding region, extending to the nearest neighboring gene. Regulatory regions can be used to construct gene fusions where the regulatory DNAs are operably fused to a coding region for

25   a reporter protein whose expression is easily detected, and these constructs are introduced as transgenes into the animal of choice. An entire regulatory DNA region can be used, or the regulatory region can be divided into smaller segments to identify sub-elements that might be specific for controlling expression a given cell type or stage of development. One suitable method to decipher regions containing regulatory sequences is by an *in vitro* CAT

30   assay (Mercer, Crit. Rev. Euk. Gene Exp. (1992) 2:251-263; Sambrook *et al.*, *supra*; and Gorman *et al.*, Mol. Cell. Biol. (1992) 2:1044-1051). Additional reporter proteins that can be used for construction of these gene fusions include *E. coli* beta-galactosidase and green fluorescent protein (GFP). These can be detected readily *in situ*, and thus are useful for histological studies and can be used to sort cells that express p53 proteins (O'Kane and

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Gehring PNAS (1987) 84(24):9123-9127; Chalfie *et al.*, Science (1994) 263:802-805; and Cumberledge and Krasnow (1994) Methods in Cell Biology 44:143-159). Recombinase proteins, such as FLP or cre, can be used in controlling gene expression through site-specific recombination (Golic and Lindquist (1989) Cell 59(3):499-509; White *et al.*, Science (1996) 271:805-807). Toxic proteins such as the reaper and hid cell death proteins, are useful to specifically ablate cells that normally express p53 proteins in order to assess the physiological function of the cells (Kingston, In Current Protocols in Molecular Biology (1998) Ausubel *et al.*, John Wiley & Sons, Inc. sections 12.0.3-12.10) or any other protein where it is desired to examine the function this particular protein specifically in cells that synthesize p53 proteins.

Alternatively, a binary reporter system can be used, similar to that described further below, where the p53 regulatory element is operably fused to the coding region of an exogenous transcriptional activator protein, such as the GAL4 or tTA activators described below, to create a p53 regulatory element "driver gene". For the other half of the binary system the exogenous activator controls a separate "target gene" containing a coding region of a reporter protein operably fused to a cognate regulatory element for the exogenous activator protein, such as UAS_G or a tTA-response element, respectively. An advantage of a binary system is that a single driver gene construct can be used to activate transcription from preconstructed target genes encoding different reporter proteins, each with its own uses as delineated above.

p53 regulatory element-reporter gene fusions are also useful for tests of genetic interactions, where the objective is to identify those genes that have a specific role in controlling the expression of p53 genes, or promoting the growth and differentiation of the tissues that expresses the p53 protein. p53 gene regulatory DNA elements are also useful in protein-DNA binding assays to identify gene regulatory proteins that control the expression of p53 genes. The gene regulatory proteins can be detected using a variety of methods that probe specific protein-DNA interactions well known to those skilled in the art (Kingston, *supra*) including *in vivo* footprinting assays based on protection of DNA sequences from chemical and enzymatic modification within living or permeabilized cells; and *in vitro* footprinting assays based on protection of DNA sequences from chemical or enzymatic modification using protein extracts, nitrocellulose filter-binding assays and gel electrophoresis mobility shift assays using radioactively labeled regulatory DNA elements mixed with protein extracts. Candidate p53 gene regulatory proteins can be purified using a combination of conventional and DNA-affinity purification techniques. Molecular cloning

strategies can also be used to identify proteins that specifically bind p53 gene regulatory DNA elements. For example, a *Drosophila* cDNA library in an expression vector, can be screened for cDNAs that encode p53 gene regulatory element DNA-binding activity. Similarly, the yeast "one-hybrid" system can be used (Li and Herskowitz, Science (1993)

5   262:1870-1874; Luo *et al.*, Biotechniques (1996) 20(4):564-568; Vidal *et al.*, PNAS (1996) 93(19):10315-10320).

Assays for tumor suppressor genes

The p53 tumor suppressor gene encodes a transcription factor implicated in

10   regulation of cell proliferation, control of the cell cycle, and induction of apoptosis. Various experimental methods may be used to assess the role of the insect p53 genes in each of these areas.

Transcription activity assays

Due to its acidic region, wild type p53 binds both specifically and non-specifically

15   to DNA in order to mediate its function (Zambetti and Levine, *supra*). Transcriptional regulation by the p53 protein or its fragments may be examined by any method known in the art. An electrophoretic mobility shift assay can be used to characterize DNA sequences to which p53 binds, and thus can assist in the identification of genes regulated by p53. Briefly, cells are grown and transfected with various amounts of wild type or mutated

20   transcription factor of interest (in this case, p53), harvested 48 hr after transfection, and lysed to prepare nuclear extracts. Preparations of *Drosophila* nuclear extracts for use in mobility shift assays may be done as described in Dignam *et al.*, Nucleic Acids Res. (1983) 11:1475-1489. Additionally, complementary, single-stranded oligonucleotides corresponding to target sequences for binding are synthesized and self-annealed to a final

25   concentration of 10-15 ng/μl. Double stranded DNA is verified by gel electrophoretic analysis (*e.g.,* on a 7% polyacrylamide gel, by methods known in the art), and end-labeled with 20 μCi [32P] γ-dATP. The nuclear extracts are mixed with the double stranded target sequences under conditions conducive for binding and the results are analyzed by polyacrylamide gel electrophoresis.

30   Another suitable method to determine DNA sequences to which p53 binds is by DNA footprinting (Schmitz *et al*, Nucleic Acids Research (1978) 5:3157-3170).

Apoptosis assays

A variety of methods may be used to examine apoptosis. One method is the terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling

(TUNEL) assay which measures the nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, Nature (1994) 371:346-347; White *et al.*, Science (1994) 264:677-683). Additionally, commercial kits can be used for detection of apoptosis (ApoAlert® available from Clontech (Palo Alto, CA).

5   Apoptosis may also be assayed by a variety of staining methods. Acridine orange can be used to detect apoptosis in cultured cells (Lucas *et al.*, Blood (1998) 15:4730-41) and in intact *Drosophila* tissues, which can also be stained with Nile Blue (Abrams *et al.*, Development (1993) 117:29-43). Another assay that can be used to detect DNA laddering employs ethidium bromide staining and electophoresis of DNA on an agarose gel (Civielli

10   *et al.*, Int. J. Cancer (1995) 27:673-679; Young, J. Biol. Chem. (1998) 273:25198-25202).

Proliferation and cell cycle assays

Proliferating cells may be identified by bromodeoxyuridine (BRDU) incorporation into cells undergoing DNA synthesis and detection by an anti-BRDU antibody (Hoshino *et al.*, Int. J. Cancer (1986) 38:369; Campana *et al.*, J. Immunol. Meth. (1988) 107:79). This

15   assay can be used to reproducibly identify S-phase cells in *Drosophila* embryos (Edgar and O'Farrell, Cell (1990) 62:469-480) and imaginal discs (Secombe *et al.*, Genetics (1998) 149:1867-1882). S-phase DNA syntheses can also be quantified by measuring [^{3}H]-thymidine incorporation using a scintillation counter (Chen, Oncogene (1996) 13:1395-403; Jeoung, J. Biol. Chem. (1995) 270:18367-73). Cell proliferation may be measured by

20   counting samples of a cell population over time, for example using a hemacytometer and Trypan-blue staining.   ¦

The DNA content and/or mitotic index of the cells may be measured based on the DNA ploidy value of the cell using a variety of methods known in the art such as a propidum iodide assay (Turner *et al.*, Prostate (1998) 34:175-81) or Feulgen staining using

25   a computerized microdensitometry staining system (Bacus, Am. J. Pathol.(1989) 135:783-92).

The effect of p53 overexpression or loss-of-function on *Drosophila* cell proliferation can be assayed *in vivo* using an assay in which clones of cells with altered gene expression are generated in the developing wing disc of *Drosophila* (Neufeld *et al.*, Cell (1998)

30   93:1183-93). The clones coexpress GFP, which allows the size and DNA content of the mutant and wild-type cells from dissociated discs to be compared by FACS analysis.

Tumor formation and transformation assays

A variety of *in vivo* and *in vitro* tumor formation assays are known in the art that can be used to assay p53 function. Such assays can be used to detect foci formation (Beenken,

J. Surg. Res. (1992) 52:401-5), *in vitro* transformation (Ginsberg, Oncogene. (1991) 6:669-72), tumor formation in nude mice (Endlich, Int. J. Radiat. Biol. (1993) 64:715-26), tumor formation in *Drosophila* (Tao *et al.*, Nat. Genet. (1999) 21:177-181), and anchorage-independent growth in soft agar (Endlich, *supra*). Loss of indicia of

5   differentiation may be indicate transformation, including loss of differentiation markers, cell rounding, loss of adhesion, loss of polarity, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, and expression of fetal antigens.

10   **Generation and Genetic Analysis of Animals and Cell Lines with Altered Expression of p53 Gene**

Both genetically modified animal models (i.e. *in vivo* models), such as *C. elegans* and *Drosophila*, and *in vitro* models such as genetically engineered cell lines expressing or mis-expressing p53 genes, are useful for the functional analysis of these proteins. Model

15   systems that display detectable phenotypes, can be used for the identification and characterization of p53 genes or other genes of interest and/or phenotypes associated with the mutation or mis-expression of p53. The term "mis-expression" as used herein encompasses mis-expression due to gene mutations. Thus, a mis-expressed p53 protein may be one having an amino acid sequence that differs from wild-type (i.e. it is a derivative

20   of the normal protein). A mis-expressed p53 protein may also be one in which one or more N- or C- terminal amino acids have been deleted, and thus is a "fragment" of the normal protein. As used herein, "mis-expression" also includes ectopic expression (*e.g.* by altering the normal spatial or temporal expression), over-expression (*e.g.* by multiple gene copies), underexpression, non-expression (*e.g.* by gene knockout or blocking expression that would

25   otherwise normally occur), and further, expression in ectopic tissues.

The *in vivo* and *in vitro* models may be genetically engineered or modified so that they 1) have deletions and/or insertions of a p53 genes, 2) harbor interfering RNA sequences derived from a p53 gene, 3) have had an endogenous p53 gene mutated (*e.g.* contain deletions, insertions, rearrangements, or point mutations in the p53 gene), and/or 4)

30   contain transgenes for mis-expression of wild-type or mutant forms of a p53 gene. Such genetically modified *in vivo* and *in vitro* models are useful for identification of genes and proteins that are involved in the synthesis, activation, control, *etc.* of p53, and also downstream effectors of p53 function, genes regulated by p53, *etc.* The model systems can be used for testing potential pharmaceutical and pesticidal compounds that interact with

p53, for example by administering the compound to the model system using any suitable method (*e.g.* direct contact, ingestion, injection, *etc.*) and observing any changes in phenotype, for example defective movement, lethality, *etc.* Various genetic engineering and expression modification methods which can be used are well-known in the art,

5   including chemical mutagenesis, transposon mutagenesis, antisense RNAi, dsRNAi, and transgene-mediated mis-expression.

Generating Loss-of-function Mutations by Mutagenesis

Loss-of-function mutations in an insect p53 gene can be generated by any of several mutagenesis methods known in the art (Ashburner, In Drosophila melanogaster: A

10   Laboratory Manual (1989), Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press: pp. 299-418; Fly pushing: The Theory and Practice of Drosophila melanogaster Genetics (1997) Cold Spring Harbor Press, Plainview, NY, hereinafter "Fly Pushing"). Techniques for producing mutations in a gene or genome include use of radiation (*e.g.*, X-ray, UV, or gamma ray); chemicals (*e.g.*, EMS, MMS, ENU, formaldehyde, *etc.*); and insertional

15   mutagenesis by mobile elements including dysgenesis induced by transposon insertions, or transposon-mediated deletions, for example, male recombination, as described below. Other methods of altering expression of genes include use of transposons (*e.g.*, P element, EP-type "overexpression trap" element, mariner element, *piggyBac* transposon, hermes, minos, sleeping beauty, *etc.*) to misexpress genes; antisense; double-stranded RNA

20   interference; peptide and RNA aptamers; directed deletions; homologous recombination; dominant negative alleles; and intrabodies.

Transposon insertions lying adjacent to a p53 gene can be used to generate deletions of flanking genomic DNA, which if induced in the germline, are stably propagated in subsequent generations. The utility of this technique in generating deletions has been

25   demonstrated and is well-known in the art. One version of the technique using collections of P element transposon induced recessive lethal mutations (P lethals) is particularly suitable for rapid identification of novel, essential genes in *Drosophila* (Cooley *et al.*, Science (1988) 239:1121-1128; Spralding *et al.*, PNAS (1995) 92:0824-10830). Since the sequence of the P elements are known, the genomic sequence flanking each transposon

30   insert is determined either by plasmid rescue (Hamilton *et al.*, PNAS (1991) 88:2731-2735) or by inverse polymerase chain reaction (Rehm, http://www.fruitfly.org/methods/). A more recent version of the transposon insertion technique in male *Drosophila* using P elements is known as P-mediated male recombination (Preston and Engels, Genetics (1996) 144:1611-1638).

Generating Loss-of-function Phenotypes Using RNA-based Methods

p53 genes may be identified and/or characterized by generating loss-of-function phenotypes in animals of interest through RNA-based methods, such as antisense RNA (Schubiger and Edgar, Methods in Cell Biology (1994) 44:697-713). One form of the antisense RNA method involves the injection of embryos with an antisense RNA that is partially homologous to the gene of interest (in this case the p53 gene). Another form of the antisense RNA method involves expression of an antisense RNA partially homologous to the gene of interest by operably joining a portion of the gene of interest in the antisense orientation to a powerful promoter that can drive the expression of large quantities of antisense RNA, either generally throughout the animal or in specific tissues. Antisense RNA-generated loss-of-function phenotypes have been reported previously for several *Drosophila* genes including cactus, pecanex, and Krüppel (LaBonne *et al.*, Dev. Biol. (1989) 136(1):1-16; Schuh and Jackle, Genome (1989) 31(1):422-425; Geisler *et al.*, Cell (1992) 71(4):613-621).

Loss-of-function phenotypes can also be generated by cosuppression methods (Bingham, Cell (1997) 90(3):385-387; Smyth, Curr. Biol. (1997) 7(12):793-795; Que and Jorgensen, Dev. Genet. (1998) 22(1):100-109). Cosuppression is a phenomenon of reduced gene expression produced by expression or injection of a sense strand RNA corresponding to a partial segment of the gene of interest. Cosuppression effects have been employed extensively in plants and *C. elegans* to generate loss-of-function phenotypes. Cosuppression in *Drosophila* has been shown, where reduced expression of the Adh gene was induced from a white-Adh transgene (Pal-Bhadra *et al.*, Cell (1997) 90(3):479-490).

Another method for generating loss-of-function phenotypes is by double-stranded RNA interference (dsRNAi). This method is based on the interfering properties of double-stranded RNA derived from the coding regions of gene, and has proven to be of great utility in genetic studies of *C. elegans* (Fire *et al.*, Nature (1998) 391:806-811), and can also be used to generate loss-of-function phenotypes in *Drosophila* (Kennerdell and Carthew, Cell (1998) 95:1017-1026; Misquitta and Patterson PNAS (1999) 96:1451-1456). Complementary sense and antisense RNAs derived from a substantial portion of a gene of interest, such as p53 gene, are synthesized *in vitro*, annealed in an injection buffer, and introduced into animals by injection or other suitable methods such as by feeding, soaking the animals in a buffer containing the RNA, *etc.* Progeny of the dsRNA treated animals are then inspected for phenotypes of interest (PCT publication no. WO99/32619).

dsRNAi can also be achieved by causing simultaneous expression *in vivo* of both sense and antisense RNA from appropriately positioned promoters operably fused to p53 sequences. Alternatively, the living food of an animal can be engineered to express sense and antisense RNA, and then fed to the animal. For example, *C. elegans* can be fed

5   engineered *E. coli*, *Drosophila* can be fed engineered baker's yeast, and insects such as *Leptinotarsa* and *Heliothis* and other plant-eating animals can be fed transgenic plants engineered to produce the dsRNA.

RNAi has also been successfully used in cultured *Drosophila* cells to inhibit expression of targeted proteins (Dixon lab, University of Michigan,

10   http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html). Thus, cell lines in culture can be manipulated using RNAi both to perturb and study the function of p53 pathway components and to validate the efficacy of therapeutic or pesticidal strategies which involve the manipulation of this pathway. A suitable protocol is described in Example 13.

15   **Generating Loss-of-function Phenotypes Using Peptide and RNA Aptamers**

Another method for generating loss-of-function phenotypes is by the use of peptide aptamers, which are peptides or small polypeptides that act as dominant inhibitors of protein function. Peptide aptamers specifically bind to target proteins, blocking their function ability (Kolonin and Finley, PNAS (1998) 95:14266-14271). Due to the highly

20   selective nature of peptide aptamers, they may be used not only to target a specific protein, but also to target specific functions of a given protein (*e.g.* transcription function). Further, peptide aptamers may be expressed in a controlled fashion by use of promoters which regulate expression in a temporal, spatial or inducible manner. Peptide aptamers act dominantly; therefore, they can be used to analyze proteins for which loss-of-function

25   mutants are not available.

Peptide aptamers that bind with high affinity and specificity to a target protein may be isolated by a variety of techniques known in the art. In one method, they are isolated from random peptide libraries by yeast two-hybrid screens (Xu *et al.*, PNAS (1997) 94:12473-12478). They can also be isolated from phage libraries (Hoogenboom *et al.*,

30   Immunotechnology (1998) 4:1-20) or chemically generated peptides/libraries.

RNA aptamers are specific RNA ligands for proteins, that can specifically inhibit protein function of the gene (Good *et al.*, Gene Therapy (1997) 4:45-54; Ellington. *et al.*, Biotechnol. Annu. Rev. (1995) 1:185-214). *In vitro* selection methods can be used to identify RNA aptamers having a selected specificity (Bell *et al.*, J. Biol. Chem. (1998)

273:14309-14314). It has been demonstrated that RNA aptamers can inhibit protein function in *Drosophila* (Shi *et al.*, Proc. Natl. Acad. Sci USA (19999) 96:10033-10038). Accordingly, RNA aptamers can be used to decrease the expression of p53 protein or derivative thereof, or a protein that interacts with the p53 protein.

5   Transgenic animals can be generated to test peptide or RNA aptamers *in vivo* (Kolonin and Finley, *supra*). For example, transgenic *Drosophila* lines expressing the desired aptamers may be generated by P element mediated transformation (discussed below). The phenotypes of the progeny expressing the aptamers can then be characterized.

Generating Loss of Function Phenotypes Using Intrabodies

10   Intracellularly expressed antibodies, or intrabodies, are single-chain antibody molecules designed to specifically bind and inactivate target molecules inside cells. Intrabodies have been used in cell assays and in whole organisms such as *Drosophila* (Chen *et al.*, Hum. Gen. Ther. (1994) 5:595-601; Hassanzadeh *et al.*, Febs Lett. (1998) 16(1, 2):75-80 and 81-86). Inducible expression vectors can be constructed with intrabodies that

15   react specifically with p53 protein. These vectors can be introduced into model organisms and studied in the same manner as described above for aptamers.

Transgenesis

Typically, transgenic animals are created that contain gene fusions of the coding regions of the p53 gene (from either genomic DNA or cDNA) or genes engineered to

20   encode antisense RNAs, cosuppression RNAs, interfering dsRNA, RNA aptamers, peptide aptamers, or intrabodies operably joined to a specific promoter and transcriptional enhancer whose regulation has been well characterized, preferably heterologous promoters/enhancers (i.e. promoters/enhancers that are non-native to the p53 genes being expressed).

Methods are well known for incorporating exogenous nucleic acid sequences into

25   the genome of animals or cultured cells to create transgenic animals or recombinant cell lines. For invertebrate animal models, the most common methods involve the use of transposable elements. There are several suitable transposable elements that can be used to incorporate nucleic acid sequences into the genome of model organisms. Transposable elements are also particularly useful for inserting sequences into a gene of interest so that

30   the encoded protein is not properly expressed, creating a "knock-out" animal having a loss-of-function phenotype. Techniques are well-established for the use of P element in *Drosophila* (Rubin and Spradling, Science (1982) 218:348-53; U.S. Pat. No. 4,670,388). Additionally, transposable elements that function in a variety of species, have been

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identified, such as PiggyBac (Thibault *et al.*, Insect Mol Biol (1999) 8(1):119-23), hobo, and hermes.

P elements, or marked P elements, are preferred for the isolation of loss-of-function mutations in *Drosophila* p53 genes because of the precise molecular mapping of these genes, depending on the availability and proximity of preexisting P element insertions for use as a localized transposon source (Hamilton and Zinn, Methods in Cell Biology (1994) 44:81-94; and Wolfner and Goldberg, Methods in Cell Biology (1994) 44:33-80). Typically, modified P elements are used which contain one or more elements that allow detection of animals containing the P element. Most often, marker genes are used that affect the eye color of *Drosophila*, such as derivatives of the *Drosophila white* or *rosy* genes (Rubin and Spradling, *supra*; and Klemenz *et al.*, Nucleic Acids Res. (1987) 15(10):3947-3959). However, in principle, any gene can be used as a marker that causes a reliable and easily scored phenotypic change in transgenic animals. Various other markers include bacterial plasmid sequences having selectable markers such as ampicillin resistance (Steller and Pirrotta, EMBO. J. (1985) 4:167-171); and *lacZ* sequences fused to a weak general promoter to detect the presence of enhancers with a developmental expression pattern of interest (Bellen *et al.*, Genes Dev. (1989) 3(9):1288-1300). Other examples of marked P elements useful for mutagenesis have been reported (Nucleic Acids Research (1998) 26:85-88; and http://flybase.bio.indiana.edu).

A preferred method of transposon mutagenesis in *Drosophila* employs the "local hopping" method (Tower *et al.* (Genetics (1993) 133:347-359). Each new P insertion line can be tested molecularly for transposition of the P element into the gene of interest (*e.g.* p53) by assays based on PCR. For each reaction, one PCR primer is used that is homologous to sequences contained within the P element and a second primer is homologous to the coding region or flanking regions of the gene of interest. Products of the PCR reactions are detected by agarose gel electrophoresis. The sizes of the resulting DNA fragments reveal the site of P element insertion relative to the gene of interest. Alternatively, Southern blotting and restriction mapping using DNA probes derived from genomic DNA or cDNAs of the gene of interest can be used to detect transposition events that rearrange the genomic DNA of the gene. P transposition events that map to the gene of interest can be assessed for phenotypic effects in heterozygous or homozygous mutant *Drosophila*.

In another embodiment, *Drosophila* lines carrying P insertions in the gene of interest, can be used to generate localized deletions using known methods (Kaiser,

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Bioassays (1990) 12(6):297-301; Harnessing the power of *Drosophila* genetics, In Drosophila melanogaster: Practical Uses in Cell and Molecular Biology, Goldstein and Fyrberg, Eds., Academic Press, Inc. San Diego, California). This is particularly useful if no P element transpositions are found that disrupt the gene of interest. Briefly, flies containing

5   P elements inserted near the gene of interest are exposed to a further round of transposase to induce excision of the element. Progeny in which the transposon has excised are typically identified by loss of the eye color marker associated with the transposable element. The resulting progeny will include flies with either precise or imprecise excision of the P element, where the imprecise excision events often result in deletion of genomic DNA

10   neighboring the site of P insertion. Such progeny are screened by molecular techniques to identify deletion events that remove genomic sequence from the gene of interest, and assessed for phenotypic effects in heterozygous and homozygous mutant *Drosophila*.

Recently a transgenesis system has been described that may have universal applicability in all eye-bearing animals and which has been proven effective in delivering

15   transgenes to diverse insect species (Berghammer *et al.*, Nature (1999) 402:370-371). This system includes: an artificial promoter active in eye tissue of all animal species, preferably containing three Pax6 binding sites positioned upstream of a TATA box (3xP3; Sheng *et al.* Genes Devel. (1997) 11:1122-1131); a strong and visually detectable marker gene, such as GFP or or other autofluorescent protein genes (Pasher *et al.*, Gene (1992) 111:229-233;

20   U.S. Pat. No. 5,491,084); and promiscuous vectors capable of delivering transgenes to a broad range of animal species, for example transposon-based vectors derived from *Hermes*, *PiggyBac*, or *mariner*, or vectors based on pantropic VSV_G-pseudotyped retroviruses (Burns *et al.*, In Vitro Cell Dev Biol Anim (1996) 32:78-84; Jordan *et al.*, Insect Mol Biol (1998) 7: 215-222; US Pat. No. 5,670,345). Since the same transgenesis system can be

25   used in a variety of phylogenetically diverse animals, comparative functional studies are greatly facilitated, which is especially helpful in evaluating new applications to pest management.

In addition to creating loss-of-function phenotypes, transposable elements can be used to incorporate p53, or fragments or derivatives thereof, as an additional gene into any

30   region of an animal's genome resulting in mis-expression (including over-expression) of the gene. A preferred vector designed specifically for misexpression of genes in transgenic *Drosophila*, is derived from pGMR (Hay *et al.*, Development (1994) 120:2121-2129), is 9Kb long, and contains: an origin of replication for *E. coli*; an ampicillin resistance gene; P element transposon 3' and 5' ends to mobilize the inserted sequences; a White marker gene;

an expression unit comprising the TATA region of hsp70 enhancer and the 3'untranslated region of α-tubulin gene. The expression unit contains a first multiple cloning site (MCS) designed for insertion of an enhancer and a second MCS located 500 bases downstream, designed for the insertion of a gene of interest. As an alternative to transposable elements,

5 homologous recombination or gene targeting techniques can be used to substitute a heterologous p53 gene or fragment or derivative for one or both copies of the animal's homologous gene. The transgene can be under the regulation of either an exogenous or an endogenous promoter element, and be inserted as either a minigene or a large genomic fragment. Gene function can be analyzed by ectopic expression, using, for example,

10 *Drosophila* (Brand *et al.*, Methods in Cell Biology (1994) 44:635- 654).

Examples of well-characterized heterologous promoters that may be used to create transgenic *Drosophila* include heat shock promoters/enhancers such as the *hsp70* and *hsp83* genes. Eye tissue specific promoters/enhancers include *eyeless* (Mozer and Benzer, Development (1994) 120:1049-1058), *sevenless* (Bowtell *et al.*, PNAS (1991) 88(15):6853-

15 6857), and *glass*-responsive promoters/enhancers (Quiring *et al.*, Science (1994) 265:785-789). Wing tissue specific enhancers/promoters can be derived from the *dpp* or *vestigal* genes (Staehling-Hampton *et al.*, Cell Growth Differ. (1994) 5(6):585-593; Kim *et al.*, Nature (1996) 382:133-138). Finally, where it is necessary to restrict the activity of dominant active or dominant negative transgenes to regions where p53 is normally active, it

20 may be useful to use endogenous p53 promoters. The ectopic expression of DMp53 in *Drosophila* larval eye using *glass*-responsive enhancer elements is described in Example 12 below.

In *Drosophila*, binary control systems that employ exogenous DNA are useful when testing the mis-expression of genes in a wide variety of developmental stage-specific and

25 tissue-specific patterns. Two examples of binary exogenous regulatory systems include the UAS/GAL4 system from yeast (Hay *et al.*, PNAS (1997) 94(10):5195-5200; Ellis *et al.*, Development (1993) 119(3):855-865), and the "Tet system" derived from *E. coli* (Bello *et al.*, Development (1998) 125:2193-2202). The UAS/GAL4 system is a well-established and powerful method of mis-expression which employs the UAS_G upstream regulatory

30 sequence for control of promoters by the yeast GAL4 transcriptional activator protein (Brand and Perrimon, Development (1993) 118(2):401-15). In this approach, transgenic *Drosophila*, termed "target" lines, are generated where the gene of interest to be mis-expressed is operably fused to an appropriate promoter controlled by UAS_G. Other transgenic *Drosophila* strains, termed "driver" lines, are generated where the GAL4 coding

region is operably fused to promoters/enhancers that direct the expression of the GAL4 activator protein in specific tissues, such as the eye, wing, nervous system, gut, or musculature. The gene of interest is not expressed in the target lines for lack of a transcriptional activator to drive transcription from the promoter joined to the gene of

5 interest. However, when the UAS-target line is crossed with a GAL4 driver line, mis-expression of the gene of interest is induced in resulting progeny in a specific pattern that is characteristic for that GAL4 line. The technical simplicity of this approach makes it possible to sample the effects of directed mis-expression of the gene of interest in a wide variety of tissues by generating one transgenic target line with the gene of interest, and

10 crossing that target line with a panel of pre-existing driver lines.

In the "Tet" binary control system, transgenic *Drosophila* driver lines are generated where the coding region for a tetracycline-controlled transcriptional activator (tTA) is operably fused to promoters/enhancers that direct the expression of tTA in a tissue-specific and/or developmental stage-specific manner. The driver lines are crossed with transgenic

15 *Drosophila* target lines where the coding region for the gene of interest to be mis-expressed is operably fused to a promoter that possesses a tTA-responsive regulatory element. When the resulting progeny are supplied with food supplemented with a sufficient amount of tetracycline, expression of the gene of interest is blocked. Expression of the gene of interest can be induced at will simply by removal of tetracycline from the food. Also, the level of

20 expression of the gene of interest can be adjusted by varying the level of tetracycline in the food. Thus, the use of the Tet system as a binary control mechanism for mis-expression has the advantage of providing a means to control the amplitude and timing of mis-expression of the gene of interest, in addition to spatial control. Consequently, if a p53 gene has lethal or deleterious effects when mis-expressed at an early stage in development, such as the

25 embryonic or larval stages, the function of the gene in the adult can still be assessed by adding tetracycline to the food during early stages of development and removing tetracycline later so as to induce mis-expression only at the adult stage.

Dominant negative mutations, by which the mutation causes a protein to interfere with the normal function of a wild-type copy of the protein, and which can result in loss-of-

30 function or reduced-function phenotypes in the presence of a normal copy of the gene, can be made using known methods (Hershkowitz, Nature (1987) 329:219-222). In the case of active monomeric proteins, overexpression of an inactive form, achieved, for example, by linking the mutant gene to a highly active promoter, can cause competition for natural substrates or ligands sufficient to significantly reduce net activity of the normal protein.

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Alternatively, changes to active site residues can be made to create a virtually irreversible association with a target.

Assays for Change in Gene Expression

5   Various expression analysis techniques may be used to identify genes which are differentially expressed between a cell line or an animal expressing a wild type p53 gene compared to another cell line or animal expressing a mutant p53 gene. Such expression profiling techniques include differential display, serial analysis of gene expression (SAGE), transcript profiling coupled to a gene database query, nucleic acid array technology,

10   subtractive hybridization, and proteome analysis (*e.g.* mass-spectrometry and two-dimensional protein gels). Nucleic acid array technology may be used to determine the genome-wide expression pattern in a normal animal for comparison with an animal having a mutation in the p53 gene. Gene expression profiling can also be used to identify other genes or proteins that may have a functional relation to p53. The genes are identified by

15   detecting changes in their expression levels following mutation, over-expression, under-expression, mis-expression or knock-out, of the p53 gene.

Phenotypes Associated With p53 Gene Mutations

After isolation of model animals carrying mutated or mis-expressed p53 genes or

20   inhibitory RNAs, animals are carefully examined for phenotypes of interest. For analysis of p53 genes that have been mutated, animal models that are both homozygous and heterozygous for the altered p53 gene are analyzed. Examples of specific phenotypes that may be investigated include lethality; sterility; feeding behavior, tumor formation, perturbations in neuromuscular function including alterations in motility, and alterations in

25   sensitivity to pharmaceuticals. Some phenotypes more specific to flies include alterations in: adult behavior such as, flight ability, walking, grooming, phototaxis, mating or egg-laying; alterations in the responses of sensory organs, changes in the morphology, size or number of adult tissues such as, eyes, wings, legs, bristles, antennae, gut, fat body, gonads, and musculature; larval tissues such as mouth parts, cuticles, internal tissues or imaginal

30   discs; or larval behavior such as feeding, molting, crawling, or puparian formation; or developmental defects in any germline or embryonic tissues.

Genomic sequences containing a p53 gene can be used to engineer an existing mutant insect line, using the transgenesis methods previously described, to determine whether the mutation is in the p53 gene. Briefly, germline transformants are crossed for

complementation testing to an existing or newly created panel of insect lines whose mutations have been mapped to the vicinity of the gene of interest (Fly Pushing, *supra*). If a mutant line is discovered to be rescued by the genomic fragment, as judged by complementation of the mutant phenotype, then the mutant line likely harbors a mutation in

5   the p53 gene. This prediction can be further confirmed by sequencing the p53 gene from the mutant line to identify the lesion in the p53 gene.

Identification of Genes That Modify p53 Genes

The characterization of new phenotypes created by mutations or misexpression in

10   p53 genes enables one to test for genetic interactions between p53 genes and other genes that may participate in the same, related, or interacting genetic or biochemical pathway(s). Individual genes can be used as starting points in large-scale genetic modifier screens as described in more detail below. Alternatively, RNAi methods can be used to simulate loss-of-function mutations in the genes being analyzed. It is of particular interest to investigate

15   whether there are any interactions of p53 genes with other well-characterized genes, particularly genes involved in regulation of the cell cycle or apoptosis.

Genetic Modifier Screens

A genetic modifier screen using invertebrate model organisms is a particularly preferred method for identifying genes that interact with p53 genes, because large numbers

20   of animals can be systematically screened making it more possible that interacting genes will be identified. In *Drosophila*, a screen of up to about 10,000 animals is considered to be a pilot-scale screen. Moderate-scale screens usually employ about 10,000 to about 50,000 flies, and large-scale screens employ greater than about 50,000 flies. In a genetic modifier screen, animals having a mutant phenotype due to a mutation in or misexpression of the p53

25   gene are further mutagenized, for example by chemical mutagenesis or transposon mutagenesis.

The procedures involved in typical *Drosophila* genetic modifier screens are well-known in the art (Wolfner and Goldberg, Methods in Cell Biology (1994) 44:33-80; and Karim *et al.*, Genetics (1996) 143:315-329). The procedures used differ depending upon

30   the precise nature of the mutant allele being modified. If the mutant allele is genetically recessive, as is commonly the situation for a loss-of-function allele, then most typically males, or in some cases females, which carry one copy of the mutant allele are exposed to an effective mutagen, such as EMS, MMS, ENU, triethylamine, diepoxyalkanes, ICR-170, formaldehyde, X-rays, gamma rays, or ultraviolet radiation. The mutagenized animals are

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crossed to animals of the opposite sex that also carry the mutant allele to be modified. In the case where the mutant allele being modified is genetically dominant, as is commonly the situation for ectopically expressed genes, wild type males are mutagenized and crossed to females carrying the mutant allele to be modified.

5   The progeny of the mutagenized and crossed flies that exhibit either enhancement or suppression of the original phenotype are presumed to have mutations in other genes, called "modifier genes", that participate in the same phenotype-generating pathway. These progeny are immediately crossed to adults containing balancer chromosomes and used as founders of a stable genetic line. In addition, progeny of the founder adult are retested

10   under the original screening conditions to ensure stability and reproducibility of the phenotype. Additional secondary screens may be employed, as appropriate, to confirm the suitability of each new modifier mutant line for further analysis.

Standard techniques used for the mapping of modifiers that come from a genetic screen in *Drosophila* include meiotic mapping with visible or molecular genetic markers;

15   male-specific recombination mapping relative to P-element insertions; complementation analysis with deficiencies, duplications, and lethal P-element insertions; and cytological analysis of chromosomal aberrations (Fly Pushing, *supra*). Genes corresponding to modifier mutations that fail to complement a lethal P-element may be cloned by plasmid rescue of the genomic sequence surrounding that P-element. Alternatively, modifier genes

20   may be mapped by phenotype rescue and positional cloning (Sambrook *et al.*, *supra*).

Newly identified modifier mutations can be tested directly for interaction with other genes of interest known to be involved or implicated with p53 genes using methods described above. Also, the new modifier mutations can be tested for interactions with genes in other pathways that are not believed to be related to regulation of cell cycle or apoptosis.

25   New modifier mutations that exhibit specific genetic interactions with other genes implicated in cell cycle regulation or apoptosis, and not with genes in unrelated pathways, are of particular interest.

The modifier mutations may also be used to identify "complementation groups". Two modifier mutations are considered to fall within the same complementation group if

30   animals carrying both mutations in trans exhibit essentially the same phenotype as animals that are homozygous for each mutation individually and, generally are lethal when in trans to each other (Fly Pushing, *supra*). Generally, individual complementation groups defined in this way correspond to individual genes.

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When p53 modifier genes are identified, homologous genes in other species can be isolated using procedures based on cross-hybridization with modifier gene DNA probes, PCR-based strategies with primer sequences derived from the modifier genes, and/or computer searches of sequence databases. For therapeutic applications related to the function of p53 genes, human and rodent homologs of the modifier genes are of particular interest.

Although the above-described *Drosophila* genetic modifier screens are quite powerful and sensitive, some genes that interact with p53 genes may be missed in this approach, particularly if there is functional redundancy of those genes. This is because the vast majority of the mutations generated in the standard mutagenesis methods will be loss-of-function mutations, whereas gain-of-function mutations that could reveal genes with functional redundancy will be relatively rare. Another method of genetic screening in *Drosophila* has been developed that focuses specifically on systematic gain-of-function genetic screens (Rorth *et al.*, Development (1998) 125:1049-1057). This method is based on a modular mis-expression system utilizing components of the GAL4/UAS system (described above) where a modified P element, termed an "enhanced P" (EP) element, is genetically engineered to contain a GAL4-responsive UAS element and promoter. Any other transposons can also be used for this system. The resulting transposon is used to randomly tag genes by insertional mutagenesis (similar to the method of P element mutagenesis described above). Thousands of transgenic *Drosophila* strains, termed EP lines, can be generated, each containing a specific UAS-tagged gene. This approach takes advantage of the preference of P elements to insert at the 5'-ends of genes. Consequently, many of the genes that are tagged by insertion of EP elements become operably fused to a GAL4-regulated promoter, and increased expression or mis-expression of the randomly tagged gene can be induced by crossing in a GAL4 driver gene.

Systematic gain-of-function genetic screens for modifiers of phenotypes induced by mutation or mis-expression of a p53 gene can be performed by crossing several thousand *Drosophila* EP lines individually into a genetic background containing a mutant or mis-expressed p53 gene, and further containing an appropriate GAL4 driver transgene. It is also possible to remobilize the EP elements to obtain novel insertions. The progeny of these crosses are then analyzed for enhancement or suppression of the original mutant phenotype as described above. Those identified as having mutations that interact with the p53 gene can be tested further to verify the reproducibility and specificity of this genetic interaction. EP insertions that demonstrate a specific genetic interaction with a mutant or mis-expressed

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p53 gene, have a physically tagged new gene which can be identified and sequenced using PCR or hybridization screening methods, allowing the isolation of the genomic DNA adjacent to the position of the EP element insertion.

Identification of Molecules that Interact With p53

A variety of methods can be used to identify or screen for molecules, such as proteins or other molecules, that interact with p53 protein, or derivatives or fragments thereof. The assays may employ purified p53 protein, or cell lines or a model organism such as *Drosophila* that has been genetically engineered to express p53 protein. Suitable screening methodologies are well known in the art to test for proteins and other molecules that interact with a gene/protein of interest (see *e.g.*, PCT International Publication No. WO 96/34099). The newly identified interacting molecules may provide new targets for pharmaceutical agents. Any of a variety of exogenous molecules, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides, or phage display libraries), may be screened for binding capacity. In a typical binding experiment, the p53 protein or fragment is mixed with candidate molecules under conditions conducive to binding, sufficient time is allowed for any binding to occur, and assays are performed to test for bound complexes. A variety of assays to find interacting proteins are known in the art, for example, immunoprecipitation with an antibody that binds to the protein in a complex followed by analysis by size fractionation of the immunoprecipitated proteins (*e.g.* by denaturing or nondenaturing polyacrylamide gel electrophoresis), Western analysis, non-denaturing gel electrophoresis, *etc.*

Two-hybrid assay systems

A preferred method for identifying interacting proteins is a two-hybrid assay system or variation thereof (Fields and Song, Nature (1989) 340:245-246; U.S. Pat. No. 5,283,173; for review see Brent and Finley, Annu. Rev. Genet. (1997) 31:663-704). The most commonly used two-hybrid screen system is performed using yeast. All systems share three elements: 1) a gene that directs the synthesis of a "bait" protein fused to a DNA binding domain; 2) one or more "reporter" genes having an upstream binding site for the bait, and 3) a gene that directs the synthesis of a "prey" protein fused to an activation domain that activates transcription of the reporter gene. For the screening of proteins that interact with p53 protein, the "bait" is preferably a p53 protein, expressed as a fusion protein to a DNA binding domain; and the "prey" protein is a protein to be tested for ability to interact with the bait, and is expressed as a fusion protein to a transcription activation

domain. The prey proteins can be obtained from recombinant biological libraries expressing random peptides.

The bait fusion protein can be constructed using any suitable DNA binding domain, such as the *E. coli* LexA repressor protein, or the yeast GAL4 protein (Bartel *et al.*, BioTechniques (1993) 14:920-924, Chasman *et al.*, Mol. Cell. Biol. (1989) 9:4746-4749; Ma *et al.*, Cell (1987) 48:847-853; Ptashne *et al.*, Nature (1990) 346:329-331). The prey fusion protein can be constructed using any suitable activation domain such as GAL4, VP-16, *etc.* The preys may contain useful moieties such as nuclear localization signals (Ylikomi *et al.*, EMBO J. (1992) 11:3681-3694; Dingwall and Laskey, Trends Biochem. Sci. Trends Biochem. Sci. (1991) 16:479-481) or epitope tags (Allen *et al.*, Trends Biochem. Sci. Trends Biochem. Sci. (1995) 20:511-516) to facilitate isolation of the encoded proteins. Any reporter gene can be used that has a detectable phenotype such as reporter genes that allow cells expressing them to be selected by growth on appropriate medium (*e.g.* HIS3, LEU2 described by Chien *et al.*, PNAS (1991) 88:9572-9582; and Gyuris *et al.*, Cell (1993) 75:791-803). Other reporter genes, such as LacZ and GFP, allow cells expressing them to be visually screened (Chien *et al.*, *supra*).

Although the preferred host for two-hybrid screening is the yeast, the host cell in which the interaction assay and transcription of the reporter gene occurs can be any cell, such as mammalian (*e.g.* monkey, mouse, rat, human, bovine), chicken, bacterial, or insect cells. Various vectors and host strains for expression of the two fusion protein populations in yeast can be used (U.S. Pat. No. 5,468,614; Bartel *et al.*, Cellular Interactions in Development (1993) Hartley, ed., Practical Approach Series xviii, IRL Press at Oxford University Press, New York, NY, pp. 153-179; and Fields and Sternglanz, Trends In Genetics (1994) 10:286-292). As an example of a mammalian system, interaction of activation tagged VP16 derivatives with a GAL4-derived bait drives expression of reporters that direct the synthesis of hygromycin B phosphotransferase, chloramphenicol acetyltransferase, or CD4 cell surface antigen (Fearon *et al.*, PNAS (1992) 89:7958-7962). As another example, interaction of VP16-tagged derivatives with GAL4-derived baits drives the synthesis of SV40 T antigen, which in turn promotes the replication of the prey plasmid, which carries an SV40 origin (Vasavada *et al.*, PNAS (1991) 88:10686-10690).

Typically, the bait p53 gene and the prey library of chimeric genes are combined by mating the two yeast strains on solid or liquid media for a period of approximately 6-8 hours. The resulting diploids contain both kinds of chimeric genes, i.e., the DNA-binding domain fusion and the activation domain fusion. Transcription of the reporter gene can be

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detected by a linked replication assay in the case of SV40 T antigen (Vasavada *et al., supra*) or using immunoassay methods (Alam and Cook, Anal. Biochem. (1990)188:245-254). The activation of other reporter genes like URA3, HIS3, LYS2, or LEU2 enables the cells to grow in the absence of uracil, histidine, lysine, or leucine, respectively, and hence serves

5 as a selectable marker. Other types of reporters are monitored by measuring a detectable signal. For example, GFP and lacZ have gene products that are fluorescent and chromogenic, respectively.

After interacting proteins have been identified, the DNA sequences encoding the proteins can be isolated. In one method, the activation domain sequences or DNA-binding

10 domain sequences (depending on the prey hybrid used) are amplified, for example, by PCR using pairs of oligonucleotide primers specific for the coding region of the DNA binding domain or activation domain. If a shuttle (yeast to *E. coli*) vector is used to express the fusion proteins, the DNA sequences encoding the proteins can be isolated by transformation of *E. coli* using the yeast DNA and recovering the plasmids from *E. coli*. Alternatively, the

15 yeast vector can be isolated, and the insert encoding the fusion protein subcloned into a bacterial expression vector, for growth of the plasmid in *E. coli*.

Antibodies and Immunoassay

p53 proteins encoded by any of SEQ ID NOs:2, 4, 6, 8, or 10 and derivatives and fragments thereof, such as those discussed above, may be used as an immunogen to

20 generate monoclonal or polyclonal antibodies and antibody fragments or derivatives (*e.g.* chimeric, single chain, Fab fragments). For example, fragments of a p53 protein, preferably those identified as hydrophilic, are used as immunogens for antibody production using art-known methods such as by hybridomas; production of monoclonal antibodies in germ-free animals (PCT/US90/02545); the use of human hybridomas (Cole *et al.*, PNAS (1983)

25 80:2026-2030; Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy (1985) Alan R. Liss, pp. 77-96), and production of humanized antibodies (Jones *et al.*, Nature (1986) 321:522-525; U.S. Pat. 5,530,101). In a particular embodiment, p53 polypeptide fragments provide specific antigens and/or immunogens, especially when coupled to carrier proteins. For example, peptides are covalently coupled to keyhole limpet antigen (KLH) and the

30 conjugate is emulsified in Freund's complete adjuvant. Laboratory rabbits are immunized according to conventional protocol and bled. The presence of specific antibodies is assayed by solid phase immunosorbent assays using immobilized corresponding polypeptide. Specific activity or function of the antibodies produced may be determined by convenient *in vitro,* cell-based, or *in vivo* assays: *e.g.* *in vitro* binding assays, *etc.* Binding affinity may be

assayed by determination of equilibrium constants of antigen-antibody association (usually at least about $10^7\,M^{-1}$, preferably at least about $10^8\,M^{-1}$, more preferably at least about 10^9 M^{-1}). Example 11 below further describes the generation of anti-DMp53 antibodies.

Immunoassays can be used to identify proteins that interact with or bind to p53 protein. Various assays are available for testing the ability of a protein to bind to or compete with binding to a wild-type p53 protein or for binding to an anti-p53 protein antibody. Suitable assays include radioimmunoassays, ELISA (enzyme linked immunosorbent assay), immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, *etc.*

Identification of Potential Drug Targets

Once new p53 genes or p53 interacting genes are identified, they can be assessed as potential drug or pesticide targets using animal models such as *Drosophila* or other insects, or using cells that express endogenous p53, or that have been engineered to express p53.

Assays of Compounds on Insects

Potential insecticidal compounds can be administered to insects in a variety of ways, including orally (including addition to synthetic diet, application to plants or prey to be consumed by the test organism), topically (including spraying, direct application of compound to animal, allowing animal to contact a treated surface), or by injection. Insecticides are typically very hydrophobic molecules and must commonly be dissolved in organic solvents, which are allowed to evaporate in the case of methanol or acetone, or at low concentrations can be included to facilitate uptake (ethanol, dimethyl sulfoxide).

The first step in an insect assay is usually the determination of the minimal lethal dose (MLD) on the insects after a chronic exposure to the compounds. The compounds are usually diluted in DMSO, and applied to the food surface bearing 0-48 hour old embryos and larvae. In addition to MLD, this step allows the determination of the fraction of eggs that hatch, behavior of the larvae, such as how they move /feed compared to untreated larvae, the fraction that survive to pupate, and the fraction that eclose (emergence of the adult insect from puparium). Based on these results more detailed assays with shorter exposure times may be designed, and larvae might be dissected to look for obvious

morphological defects. Once the MLD is determined, more specific acute and chronic assays can be designed.

In a typical acute assay, compounds are applied to the food surface for embryos, larvae, or adults, and the animals are observed after 2 hours and after an overnight

5   incubation. For application on embryos, defects in development and the percent that survive to adulthood are determined. For larvae, defects in behavior, locomotion, and molting may be observed. For application on adults, behavior and neurological defects are observed, and effects on fertility are noted. Any deleterious effect on insect survival, motility and fertility indicates that the compound has utility in controlling pests.

10   For a chronic exposure assay, adults are placed on vials containing the compounds for 48 hours, then transferred to a clean container and observed for fertility, neurological defects, and death.

Assay of Compounds using Cell Cultures

Compounds that modulate (*e.g.* block or enhance) p53 activity may be tested on

15   cells expressing endogenous normal or mutant p53s, and/or on cells transfected with vectors that express p53, or derivatives or fragments of p53. The compounds are added at varying concentration and their ability to modulate the activity of p53 genes is determined using any of the assays for tumor suppressor genes described above (*e.g.* by measuring transcription activity, apoptosis, proliferation/cell cycle, and/or transformation). Compounds that

20   selectively modulate p53 are identified as potential drug candidates having p53 specificity.

Identification of small molecules and compounds as potential pharmaceutical compounds from large chemical libraries requires high-throughput screening (HTS) methods (Bolger, Drug Discovery Today (1999) 4:251-253). Several of the assays mentioned herein can lend themselves to such screening methods. For example, cells or

25   cell lines expressing wild type or mutant p53 protein or its fragments, and a reporter gene can be subjected to compounds of interest, and depending on the reporter genes, interactions can be measured using a variety of methods such as color detection, fluorescence detection (*e.g.* GFP), autoradiography, scintillation analysis, *etc.*

30   ### Agricultural uses of insect p53 sequences

Insect p53 genes may be used in controlling agriculturally important pest species. For example, the proteins, genes, and RNAs disclosed herein, or their fragments may have activity in modifying the growth, feeding and/or reproduction of crop-damaging insects, or insect pests of farm animals or of other animals. In general, effective pesticides exert a

disabling activity on the target pest such as lethality, sterility, paralysis, blocked development, or cessation of feeding. Such pests include egg, larval, juvenile and adult forms of flies, mosquitos, fleas, moths, beetles, cicadia, grasshoppers, aphids and crickets. The functional analyses of insect p53 genes described herein has revealed roles for these

5   genes and proteins in controlling apoptosis, response to DNA damaging agents, and protection of cells of the germline. Since overexpression of DMp53 induces apoptosis in *Drosophila*, the insect p53 genes and proteins in an activated form have application as "cell death" genes which if delivered to or expressed in specific target tissues such as the gut, nervous system, or gonad, would have a use in controlling insect pests. Alternatively, since

10 DMp53 plays a role in response to DNA damaging agents such as X-rays, interference with p53 function in insects has application in sensitizing insects to DNA damaging agents for sterilization. For example, current methods for controlling pest populations through the release of irradiated insects into the environment (Knipling, J Econ Ent (1955) 48: 459-462; Knipling (1979) U.S. Dept. Agric. Handbook No. 512) could be improved by causing

15 expression of dominant negative forms of p53 genes, proteins, or RNAs in insects and most preferably germline tissue of insects, or by exposing insects to chemical compounds which block p53 function.

Mutational analysis of insect p53 proteins may also be used in connection with the control of agriculturally-important pests. In this regard, mutational analysis of insect p53

20 genes provides a rational approach to determine the precise biological function of this class of proteins in invertebrates. Further, mutational analysis coupled with large-scale systematic genetic modifier screens provides a means to identify and validate other potential pesticide targets that might be constituents of the p53 signaling pathway. Tests for pesticidal activities can be any method known in the art. Pesticides comprising

25 the nucleic acids of the insect p53 proteins may be prepared in a suitable vector for delivery to a plant or animal. Such vectors include *Agrobacterium tumefaciens* Ti plasmid-based vectors for the generation of transgenic plants (Horsch *et al.*, Proc Natl Acad Sci U S A. (1986) 83(8):2571-2575; Fraley *et al.*, Proc. Natl. Acad. Sci. USA (1983) 80:4803) or recombinant cauliflower mosaic virus for the incoulation of plant cells or plants (U.S. Pat

30 No. 4,407,956); retrovirus based vectors for the introduction of genes into vertebrate animals (Burns *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90:8033-37); and vectors based on transposable elements for incorporation into invertebrate animals using vectors and methods already described above. For example, transgenic insects can be generated using a transgene comprising a p53 gene operably fused to an appropriate inducible promoter, such

as a tTA-responsive promoter, in order to direct expression of the tumor suppressor protein at an appropriate time in the life cycle of the insect. In this way, one may test efficacy as an insecticide in, for example, the larval phase of the life cycle (*e.g.,* when feeding does the greatest damage to crops).

5   Recombinant or synthetic p53 proteins, RNAs or their fragments, in wild-type or mutant forms, can be assayed for insecticidal activity by injection of solutions of p53 proteins or RNAs into the hemolymph of insect larvae (Blackburn, *et al.*, Appl. Environ. Microbiol. (1998) 64(8):3036-41; Bowen and Ensign, Appl. Environ. Microbiol. (1998) 64(8):3029-35). Further, transgenic plants that express p53 proteins or RNAs or their

10   fragments can be tested for activity against insect pests (Estruch *et al.*, Nat. Biotechnol. (1997) 15(2):137-41).

Insect p53 genes may be used as insect control agents in the form of recombinant viruses that direct the expression of a tumor suppressor gene in the target pest. A variety of suitable recombinant virus systems for expression of proteins in infected insect cells are

15   well known in the art. A preferred system uses recombinant baculoviruses. The use of recombinant baculoviruses as a means to engineer expression of toxic proteins in insects, and as insect control agents, has a number of specific advantages including host specificity, environmental safety, the availability of vector systems, and the potential use of the recombinant virus directly as a pesticide without the need for purification or formulation of

20   the tumor suppressor protein (Cory and Bishop, Mol. Biotechnol. (1997) 7(3):303-13; and U.S. Pat. Nos. 5,470,735; 5,352,451; 5, 770, 192; 5,759,809; 5,665,349; and 5,554,592). Thus, recombinant baculoviruses that direct the expression of insect p53 genes can be used for both testing the pesticidal activity of tumor suppressor proteins under controlled laboratory conditions, and as insect control agents in the field. One disadvantage of wild

25   type baculoviruses as insect control agents can be the amount of time between application of the virus and death of the target insect, typically one to two weeks. During this period, the insect larvae continue to feed and damage crops. Consequently, there is a need to develop improved baculovirus-derived insect control agents which result in a rapid cessation of feeding of infected target insects. The cell cycle and apoptotic regulatory roles

30   of p53 in vertebrates raises the possibility that expression of tumor suppressor proteins from recombinant baculovirus in infected insects may have a desirable effect in controlling metabolism and limiting feeding of insect pests.

Insect p53 genes, RNAs, proteins or fragments may be formulated with any carrier suitable for agricultural use, such as water, organic solvents and/or inorganic solvents. The

pesticide composition may be in the form of a solid or liquid composition and may be prepared by fundamental formulation processes such as dissolving, mixing, milling, granulating, and dispersing. Compositions may contain an insect p53 protein or gene in a mixture with agriculturally acceptable excipients such as vehicles, carriers, binders, UV

5   blockers, adhesives, hemecants, thickeners, dispersing agents, preservatives and insect attractants. Thus the compositions of the invention may, for example, be formulated as a solid comprising the active agent and a finely divided solid carrier. Alternatively, the active agent may be contained in liquid compositions including dispersions, emulsions and suspensions thereof. Any suitable final formulation may be used, including for example,

10   granules, powder, bait pellets (a solid composition containing the active agent and an insect attractant or food substance), microcapsules, water dispersible granules, emulsions and emulsified concentrates. Examples of adjuvant or carriers suitable for use with the present invention include water, organic solvent, inorganic solvent, talc, pyrophyllite, synthetic fine silica, attapugus clay, kieselguhr chalk, diatomaceous earth, lime, calcium carbonate,

15   bontonite, fuller's earth, cottonseed hulls, wheat flour, soybean flour, pumice, tripoli, wood flour, walnut shell flour, redwood flour, and lignin. The compositions may also include conventional insecticidal agents and/or may be applied in conjunction with conventional insecticidal agents.

20   **<u>EXAMPLES</u>**

The following examples describe the isolation and cloning of the nucleic acid sequence of SEQ ID NOs:1, 3, 5, 7, 9, and 18, and how these sequences, derivatives and fragments thereof, and gene products can be used for genetic studies to elucidate mechanisms of the p53 pathway as well as the discovery of potential pharmaceutical agents

25   that interact with the pathway.

These Examples are provided merely as illustrative of various aspects of the invention and should not be construed to limit the invention in any way.

<u>Example 1: Preparation of *Drosophila* cDNA Library</u>

30   A *Drosophila* expressed sequence tag (EST) cDNA library was prepared as follows. Tissue from mixed stage embryos (0-20 hour), imaginal disks and adult fly heads were collected and total RNA was prepared. Mitochondrial rRNA was removed from the total RNA by hybridization with biotinylated rRNA specific oligonucleotides and the resulting RNA was selected for polyadenylated mRNA. The resulting material was then used to

construct a random primed library. First strand cDNA synthesis was primed using a six

nucleotide random primer. The first strand cDNA was then tailed with terminal transferase

to add approximately 15 dGTP molecules. The second strand was primed using a primer

which contained a Not1 site followed by a 13 nucleotide C-tail to hybridize to the G-tailed

5   first strand cDNA. The double stranded cDNA was ligated with BstX1 adaptors and

digested with Not1. The cDNA was then fractionated by size by electrophoresis on an

agarose gel and the cDNA greater than 700 bp was purified. The cDNA was ligated with

Not1, BstX1 digested pCDNA-sk+ vector (a derivative of pBluescript, Stratagene) and used

to transform E. coli (XL1blue). The final complexity of the library was 6×10^6

10   independent clones.

The cDNA library was normalized using a modification of the method described by

Bonaldo et al. (Genome Research (1996) 6:791-806). Biotinylated driver was prepared

from the cDNA by PCR amplification of the inserts and allowed to hybridize with single

stranded plasmids of the same library. The resulting double-stranded forms were removed

15   using strepavidin magnetic beads, the remaining single stranded plasmids were converted to

double stranded molecules using Sequenase (Amersham, Arlington Hills, IL), and the

plasmid DNA stored at -20°C prior to transformation. Aliquots of the normalized plasmid

library were used to transform E. coli (XL1blue or DH10B), plated at moderate density, and

the colonies picked into a 384-well master plate containing bacterial growth media using a

20   Qbot robot (Genetix, Christchurch, UK). The clones were allowed to grow for 24 hours at

37° C then the master plates were frozen at -80° C for storage. The total number of

colonies picked for sequencing from the normalized library was 240,000. The master plates

were used to inoculate media for growth and preparation of DNA for use as template in

sequencing reactions. The reactions were primarily carried out with primer that initiated at

25   the 5' end of the cDNA inserts. However, a minor percentage of the clones were also

sequenced from the 3' end. Clones were selected for 3' end sequencing based on either

further biological interest or the selection of clones that could extend assemblies of

contiguous sequences ("contigs") as discussed below. DNA sequencing was carried out

using ABI377 automated sequencers and used either ABI FS, dirhodamine or BigDye

30   chemistries (Applied Biosystems, Inc., Foster City, CA).

Analysis of sequences was done as follows: the traces generated by the automated

sequencers were base-called using the program "Phred" (Gordon, Genome Res. (1998)

8:195-202), which also assigned quality values to each base. The resulting sequences were

trimmed for quality in view of the assigned scores. Vector sequences were also removed. Each sequence was compared to all other fly EST sequences using the BLAST program and a filter to identify regions of near 100% identity. Sequences with potential overlap were then assembled into contigs using the programs "Phrap", "Phred" and "Consed" (Phil

5   Green, University of Washington, Seattle, Washington; http://bozeman.mbt.washington.edu/phrap.docs/phrap.html). The resulting assemblies were then compared to existing public databases and homology to known proteins was then used to direct translation of the consensus sequence. Where no BLAST homology was available, the statistically most likely translation based on codon and hexanucleotide preference was

10   used. The Pfam (Bateman *et al.*, Nucleic Acids Res. (1999) 27:260-262) and Prosite (Hofmann *et al.*, Nucleic Acids Res. (1999) 27(1):215-219) collections of protein domains were used to identify motifs in the resulting translations. The contig sequences were archived in an Oracle-based relational database (FlyTag™, Exelixis Pharmaceuticals, Inc., South San Francisco, CA).

15

Example 2: Other cDNA libraries

A *Leptinotarsa* (Colorado Potato Beetle) library was prepared using the Lambda ZAP cDNA cloning kit from Stratagene (Stratagene, La Jolla, CA, cat#200450), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt

20   primed using mRNA from mixed stage larvae *Leptinotarsa*.

A *Tribolium* library was made using pSPORT cDNA library construction system (Life Technologies, Gaithersburg, MD), following manufacturer's protocols. The original cDNA used to construct the library was oligo-dt primed using mRNA from adult *Tribolium*.

Example 3: Cloning of the p53 nucleic acid from *Drosophila* (DMp53)

25   The TBLASTN program (Altschul et al., *supra*) was used to query the FlyTag™ database with a squid p53 protein sequence (GenBank gi:1244762), chosen because the squid sequence was one of only two members of the p53 family that had been identified previously from an invertebrate. The results revealed a single sequence contig, which was

30   960 bp in length and which exhibited highly significant homology to squid p53 (score=192, $P=5.1 \times 10^{-12}$). Further analysis of this sequence with the BLASTX program against GenBank protein sequences demonstrated that this contig exhibited significant homology to the entire known family of p53-like sequences in vertebrates, and that it contained coding

sequences homologous to the p53 family that encompassed essentially all of the DNA-binding domain, which is the most conserved region of the p53 protein family. Inspection of this contig indicated that it was an incomplete cDNA, missing coding regions C-terminal to the presumptive DNA-binding domain as well as the 3' untranslated region of the mRNA.

5   The full-length cDNA clone was produced by Rapid Amplification of cDNA ends (RACE; Frohman *et al.*, PNAS (1988) 85:8998-9002). A RACE-ready library was generated from Clontech (Palo Alto, CA) *Drosophila* embryo poly A$^+$RNA (Cat#694-1) using Clontech's Marathon cDNA amplification kit (Cat# K1802), and following manufacturer's directions. The following primers were used on the library to retrieve full-

10   length clones:

3'373   CCATGCTGAAGCAATAACCACCGATG   SEQ ID NO:11

3'510   GGAACACACGCAAATTAAGTGGTTGGATGG   SEQ ID NO:12

3'566   TGATTTTGACAGCGGACCACGGG   SEQ ID NO:13

15   3'799   GGAAGTTTCTTTTCGCCCGATACACGAG   SEQ ID NO:14

5'164   GGCACAAAGAAAGCACTGATTCCGAGG   SEQ ID NO:15

5'300   GGAATCTGATGCAGTTCAGCCAGCAATC   SEQ ID NO:16

5'932   GGATCGCATCCAAGACGAACGCC   SEQ ID NO:17

20   RACE reactions to obtain additional 5' and 3' sequence of the *Drosophila* p53 cDNA were performed as follows. Each RACE reaction contained: 40 μl of H$_2$O, 5 μl of 10XAdvantage PCR buffer (Clontech), 1 μl of specific p53 RACE primer at 10 μM, 1 μl of AP1 primer (from Clontech Marathon kit) at 10 μM, 1 μl of cDNA, 1 μl of dNTPs at 5 mM, 1 μl of Advantage DNA polymerase (Clontech). For 5' RACE, the reactions

25   contained either the 3'373, 3'510, 3'566, or 3'799 primers. For 3' RACE, the reactions contained either the 5'164 or 5'300 primers. The reaction mixtures were subjected to the following thermocycling program steps for touchdown PCR: (1) 94°C 1 min, (2) 94°C 0.5 min, (3) 72°C 4 min, (4) repeat steps 2-3 four times, (5) 94°C 0.5 min, (6) 70°C 4 min, (7) repeat steps 5-6 four times, (8) 94°C 0.33 min, (9) 68°C 4 min, (10) repeat steps 8-9 24

30   times, (11) 68°C 4 min, (12) remain at 4°C.

Products of the RACE reactions were analyzed by gel electrophoresis. Discrete DNA species of the following sizes were observed in the RACE products produced with each of the following primers: 3'373, approx. 400 bp; 3'510, approx. 550 bp, 3'566, approx. 600 bp; 3'799, approx. 850 bp; 5'164, approx. 1400 bp, 5'300 approx. 1300 bp. The RACE

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DNA products were cloned directly into the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, California) following the manufacturers directions. Colonies of transformed *E. coli* were picked for each construct, and plasmid DNA prepared using a QIAGEN tip 20 kit (QIAGEN, Valencia, California). Sequences of the RACE cDNA

5   inserts in within each clone were determined using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc. Foster City, California) and either M13 reverse or BigT7 primers for priming from flanking vector sequences, or 5'932 or 3'373 primers (described above) for priming internally from *Drosophila* p53 cDNA sequences. The products were analyzed using ABI 377 DNA sequencer. Sequences were assembled

10   into a contig using the Sequencher program (Gene Codes Corporation), and contained a single open reading frame encoding a predicted protein of 385 amino acids, which compared favorably with the known lengths of vertebrate p53 proteins, 363 to 396 amino acids (Soussi *et al.*, Oncogene (1990) 5:945-952). Analysis of the predicted *Drosophila* p53 protein using the BLASTP homology searching program and the GenBank database

15   confirmed that this protein was a member of the p53 family, since it exhibited highly significant homology to all known p53 related proteins, but no significant homology to other protein families.

Example 4: Cloning of p53 Nucleic Acid Sequences from other insects

20   The PCR conditions used for cloning the p53 nucleic acid sequences comprised a denaturation step of 94° C, 5 min; followed by 35 cycles of: 94° C 1 min, 55° C 1 min 72° C 1 min; then, a final extension at 72° C 10 min. All DNA sequencing reactions were performed using standard protocols for the BigDye sequencing reagents (Applied Biosystems, Inc.) and products were analyzed using ABI 377 DNA sequencers. Trace data

25   obtained from the ABI 377 DNA sequencers was analyzed and assembled into contigs using the Phred-Phrap programs.

         The DMp53 DNA and protein sequences were used to query sequences from *Tribolium, Leptinotarsa, and Heliothis* cDNA libraries using the BLAST computer program, and the results revealed several candidate cDNA clones that might encode p53

30   related sequences. For each candidate p53 cDNA clone, well-separated, single colonies were streaked on a plate and end-sequenced to verify the clones. Single colonies were picked and the plasmid DNA was purified using Qiagen REAL Preps (Qiagen, Inc., Valencia, CA). Samples were then digested with appropriate enzymes to excise insert from

vector and determine size. For example, the vector pOT2,
(www.fruitfly.org/EST/pOT2vector.html) can be excised with Xhol/EcoRI; or pBluescript
(Stratagene) can be excised with BssH II. Clones were then sequenced using a combination
of primer walking and *in vitro* transposon tagging strategies.

5   For primer walking, primers were designed to the known DNA sequences in the
clones, using the Primer-3 software (Steve Rozen, Helen J. Skaletsky (1998) Primer3.
Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.).
These primers were then used in sequencing reactions to extend the sequence until the full
sequence of the insert was determined.

10   The GPS-1 Genome Priming System *in vitro* transposon kit (New England Biolabs,
Inc., Beverly, MA) was used for transposon-based sequencing, following manufacturer's
protocols. Briefly, multiple DNA templates with randomly interspersed primer-binding
sites were generated. These clones were prepared by picking 24 colonies/clone into a
Qiagen REAL Prep to purify DNA and sequenced by using supplied primers to perform
15   bidirectional sequencing from both ends of transposon insertion.

          Sequences were then assembled using Phred/Phrap and analyzed using Consed.
Ambiguities in the sequence were resolved by resequencing several clones. This effort
resulted in several contiguous nucleotide sequences. For *Leptinotarsa,* a contig was
assembled of 2601 bases in length, encompassing an open reading frame (ORF) of 1059
20   nucleotides encoding a predicted protein of 353 amino acids. The ORF extends from base
121-1180 of SEQ ID NO:3. For *Tribolium,* a contig was assembled of 1292 bases in length,
encompassing an ORF of 1050 nucleotides, extending from base 95-1145 of SEQ ID NO:5,
and encoding a predicted protein of 350 amino acids. The analysis of another candidate
Tribolium p53 clone also generated a second contig of 509 bases in length, encompassing a
25   partial ORF of 509 nucleotides (SEQ ID NO: 7), and encoding a partial protein of 170
amino acids. For *Heliothis,* a contig was assembled of 434 bases in length, encompassing a
partial ORF of 434 nucleotides (SEQ ID NO:9), and encoding a partial protein of 145
amino acids.

30   **Example 5: Northern Blot analysis of DMp53**
          Northern blot analysis using standard methods was performed using three different
poly(A)+ mRNA preparations, 0-12 h embryo, 12-24 h embryo, and adult, which were
fractionated on an agarose gel along with size standards and blotted to a nylon membrane.
A DNA fragment containing the entire *Drosophila* p53 coding region was excised by

HincII digestion, separated by electrophoresis in an agarose gel, extracted from the gel, and
^{32}P-labeled by random-priming using the Rediprime labeling system (Amersham,
Piscataway, NJ). Hybridization of the labeled probe to the mRNA blot was performed
overnight. The blot was washed at high stringency (0.2x SSC/0.1% SDS at 65°C) and

5   mRNA species that specifically hybridized to the probe were detected by autoradiography
using X-ray film. The results showed a single cross-hybridizing mRNA species of
approximately 1.6 kilobases in all three mRNA sources. This data was consistent with the
observed sizes of the 5' and 3' RACE products described above.

10   **Example 6: Cytogenetic mapping of the DMp53 gene**

It was of interest to identify the map location of the DMp53 gene in order to
determine whether any existing *Drosophila* mutants correspond to mutations in the DMp53
gene, as well as for engineering new mutations within this gene. The cytogenetic location
of the DMp53 gene was determined by *in situ* hybridization to polytene chromosomes

15   (Pardue, Meth Cell Biol (1994) 44:333-351) following the protocol outlined below (steps
A-C).

(A) Preparation of polytene chromosome squashes: Dissected salivary glands were
placed into a drop of 45% acetic acid. Glands were transferred to drop of 1:2:3 mixture of
lactic acid: water:acetic acid. Glands were then squashed between a cover slip and a slide

20   and incubated at 4°C overnight. Squashes were frozen in liquid N_2 and the coverslip
removed. Slides were then immediately immersed in 70% ethanol for 10 min. and then air
dried. Slides were then heat treated for 30 min. at 68°C in 2x SSC buffer. Squashes were
then dehydrated by treatment with 70% ethanol for 10 min. followed by 95% ethanol for 5
min.

25   (B) Preparation of a biotinylated hybridization probe: a solution was prepared by
mixing: 50 µl of 1 M Tris-HCl pH 7.5, 6.35 µl of 1 M $MgCl_2$, 0.85 µl of beta-
mercaptoethanol, 0.625 µl of 100 mM dATP, 0.625 µl of 100 mM dCTP, 0.625 µl of 100
mM dGTP, 125 µl of 2 M HEPES pH 6.6, and 75 µl of 10 mg/ml pd(N)$_6$ (Pharmacia,
Kalamazoo, MI). 10 µl of this solution was then mixed with 2 µl 10 mg/ml bovine serum

30   albumin, 33 µl containing (0.5 µg) DMp53 cDNA fragment denatured by quick boiling, 5 µl
of 1 mM biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN), and 1 µl of Klenow
DNA polymerase (2 U) (Boehringer Mannheim). The mixture was incubated at room
temperature overnight and the following components were then added:1 µl of 1 mg/ml
sonicated denatured salmon sperm DNA, 5.5 µl 3 M sodium acetate pH 5.2, and 150 µl

ethanol (100%). After mixing the solution was stored at -70°C for 1-2 hr. DNA precipitate was collected by centrifugation in a microcentrifuge and the pellet was washed once in 70% ethanol, dried in a vacuum, dissolved in 50 µl TE buffer, and stored at -20°C.

(C) Hybridization and staining was performed as follows: 20 µl of the probe added to a hybridization solution (112.5 µl formamide; 25 µl 20x SSC, pH 7.0; 50 µl 50% dextran sulfate; 62.5 µl distilled H_2O) was placed on the squash. A coverslip (22 mm^2) was placed on the squash and sealed with rubber cement and placed on the airtight moist chamber overnight at 42°C. Rubber cement was removed by pealing off cement, then coverslip removed in 2x SSC buffer at 37°C. Slides were washed twice 15 min each in 2x SSC buffer at 37°C. Slides were then washed twice 15 min each in PBS buffer at room temperature. A mixture of the following "Elite" solution was prepared by mixing:1 ml of PBT buffer (PBS buffer with 0.1% Tween 20), 10 µl of Vectastain A (Vector Laboratories, Burlingame, CA), and 10 µl of Vectastain B (Vector Laboratories). The mixture was then allowed to incubate for 30 min. 50 µl of the Elite solution was added to the slide then drained off. 75 µl of the Elite solution was added to slide and a coverslip was placed onto the slide. The slide was incubated in moist chamber 1.5-2 hr at 37°C. The coverslip was then removed in PBS buffer, and the slide was washed twice 10 min each in PBS buffer.

A fresh solution of DAB (diaminobenzidine) in PBT buffer was made by mixing 1 µl of 0.3% hydrogen peroxide with 40 µl 0.5 mg/ml DAB solution. 40 µl of the DAB/peroxide solution was then placed onto each slide. A coverslip was placed onto the slide and incubated 2 min. Slides were then examined under a phase microscope and reaction was stopped in PBS buffer when signal was determined to be satisfactory. Slides were then rinsed in running H_2O for 10 min. and air dried. Finally, slides were inspected under a compound microscope to assign a chromosomal location to the hybridization signal. A single clear region of hybridization was observed on the polytene chromosome squashes which was assigned to cytogenetic bands 94D2-6.

Example 7: Isolation and sequence analysis of a genomic clone for the DMp53 gene

PCR was used to generate DNA probes for identification of genomic clones containing the DMp53 gene. Each reaction (50 µl total volume) contained 100 ng *Drosophila* genomic DNA, 2.5 µM each dNTP, 1.5 mM MgCl$_2$, 2 µM of each primer, and 1 µl of TAKARA exTaq DNA polymerase (PanVera Corp., Madison, WI). Reactions were set up with primer pair 5'164 & 3'510 (described above), and thermocycling conditions used were as follows (where 0:00 indicates time in minutes:seconds): initial denaturation of

50

94°C, 2:00; followed by 10 cycles of 94°C, 0:30, 58°C 0:30, 68°C, 4:00; followed by 20 cycles of 94°C, 0:30, 55°C, 0:30, 68°C, 4:00 + 0:20 per cycle. PCR products were then fractionated by agarose gel electrophoresis, ^{32}P-labeled by nick translation, and hybridized to nylon membranes containing high-density arrayed P1 clones from the Berkeley

5   *Drosophila* Genome Project (University of California, Berkeley, and purchased from Genome Systems, Inc., St. Louis, MO). Four positive P1 clones were identified: DS01201, DS02942, DS05102, and DS06254, and each clone was verified using a PCR assay with the primer pair described above. To prepare DNA for sequencing, *E. coli* containing each P1 clone was streaked to single colonies on LB agar plates containing 25 µg/ml kanamycin,

10   and grown overnight at 37°C. Well-separated colonies for each P1 clone were picked and used to inoculate 250 ml LB medium containing 25 µg/ml kanamycin and cultures were grown for 16 hours at 37°C with shaking. Bacterial cells were collected by centrifugation, and DNA purified with a Qiagen Maxi-Prep System kit (QIAGEN, Inc., Valencia, California). Genomic DNA sequence from the P1 clones was obtained using a strategy that

15   combined shotgun and directed sequencing of a small insert plasmid DNA library derived from the P1 clone DNAs (Ruddy *et al*. Genome Research (1997) 7:441-456). All DNA sequencing and analysis were performed as descibed before, and P1 sequence contigs were analyzed using the BLAST sequence homology searching programs to identify those that contained the DMp53 gene or other coding regions. This analysis demonstrated that the

20   DMp53 gene was divided into 8 exons and 7 introns. In addition, the BLAST analysis indicated the presence of two additional genes that flank the DMp53 gene; one exhibited homology to a human gene implicated in nephropathic cystinosis (labeled CTNS-like gene) and the second gene exhibited homology to a large family of oxidoreductases. Thus, we could operationally define the limits of the DMp53 gene as an 8,805 bp corresponding the

25   DNA region lying between the putative CTNS-like and oxidoreductase-like genes.

Example 8: Analysis of p53 Nucleic Acid Sequences

Upon completion of cloning, the sequences were analyzed using the Pfam and Prosite programs, and by visual analysis and comparison with other p53 sequences.

30   Regions of cDNA encoding the various domains of SEQ ID Nos 1-6 are depicted in Table I above. Additionally, Pfam predicted p53 similarity regions for the partial TRIB-Bp53 at amino acid residues 118-165 (SEQ ID NO:8) encoded by nucleotides 354-495 (SEQ ID NO:7), and for the partial HELIOp53 at amino acid residues 105-138 (SEQ ID NO:10) encoded by nucleotides 315-414 (SEQ ID NO:9).

Nucleotide and amino acid sequences for each of the p53 nucleic acid sequences and their encoded proteins were searched against all available nucleotide and amino acid sequences in the public databases, using BLAST (Altschul *et al.*, *supra*). Tables 2-6 below summarize the results. The 5 most similar sequences are listed for each p53 gene.

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TABLE 2 – DMp53

DNA BLAST of SEQ ID NO:1	
GI#	**DESCRIPTION**
6664917= C019980	*Drosophila melanogaster*, *** SEQUENCING IN PROGRESS ***, in ordered pieces
5670489=AC008200	*Drosophila melanogaster* chromosome 3 clone BACR17P04 (D757) RPCI-98 17.P.4 map 94D-94E strain y; cn bw sp, *** SEQUENCING IN PROGRESS***, 70 unordered pieces.
4419483=AI516383	*Drosophila melanogaster* cDNA clone LD42237 5prime, mRNA sequence
4420516=AI517416	Drosophila melanogaster cDNA clone GH28349 5prime, mRNA sequence
4419333=AI516233	Drosophila melanogaster cDNA clone LD42031 5prime, mRNA sequence
PROTEIN BLAST of SEQ ID NO:2	
GI#	**DESCRIPTION**
1244764= AA98564	p53 tumor suppressor homolog *[Loligo forbesi]*
1244762= AA98563	p53 tumor suppressor homolog *[Loligo forbesi]*
2828704= AC31133	tumor protein p53 *[Xiphophorus helleri]*
2828706= AC31134	tumor protein p53 *[Xiphophorus maculatus]*
3695098= AC62643	DN p63 beta *[Mus musculus]*

TABLE 3 – CPBp53

DNA BLAST of SEQ ID NO:3	
GI#	**DESCRIPTION**
6468070= AC008132	*Homo sapiens*, complete sequence Chromosome 22q11 PAC Clone pac995o6 In CES-DGCR Region
4493931= AL034556	*Plasmodium falciparum* MAL3P5, complete sequence
3738114= AC004617	*Homo sapiens* chromosome Y, clone 264,M,20, complete sequence
4150930= AC005083	*Homo sapiens* BAC clone CTA-281G5 from 7p15-p21, complete sequence
4006838= AC006079	*Homo sapiens* chromosome 17, clone hRPK.855_D_21, complete sequence
PROTEIN BLAST of SEQ ID NO:4	
GI#	**DESCRIPTION**
1244764= AA98564	p53 tumor suppressor homolog *[Loligo forbesi]*
1244762= AA98563	p53 tumor suppressor homolog *[Loligo forbesi]*
4530686=AA03817	unnamed protein product *[unidentified]*

4803651=CAA72225	P73 splice variant *[Cercopithecus aethiops]*
2370177=CAA72219	first splice variant *[Homo sapiens]*

TABLE 4 – TRIB-Ap53

DNA BLAST of SEQ ID NO:5	
GI#	**DESCRIPTION**
5877734=AW024204	wv01h01.x1 NCI_CGAP_Kid3 *Homo sapiens* cDNA clone IMAGE:2528305 3', mRNA sequence
16555= X65053	*A.thaliana* mRNA for eukaryotic translation initiation factor 4A-2
6072079=AW101398	sd79d06.y1 Gm-c1009 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1009-612 5', mRNA sequence
6070492=AW099879	sd17g11.y2 Gm-c1012 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1012-2013 5', mRNA sequence
4105775= AF049919	*Petunia x hybrida* PGP35 (PGP35) mRNA, complete cds.
PROTEIN BLAST of SEQ ID NO:6	
GI#	**DESCRIPTION**
1244764=AAA98564	p53 tumor suppressor homolog *[Loligo forbesi]*
3273745=AAC24830	p53 homolog *[Homo sapiens]*
1244762=AAA98563	p53 tumor suppressor homolog *[Loligo forbesi]*
3695096=AAC62642	N p63 gamma *[Mus musculus]*
3695080=AAC62634	DN p63 gamma *[Homo sapiens]*

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TABLE 5 – TRIB-Bp53

DNA BLAST of SEQ ID NO:7	
GI#	**DESCRIPTION**
4689085= AF043641	*Barbus barbus* p73 mRNA, complete cds
4530689= A64588	Sequence 7 from Patent WO9728186
N/A	No further homologies
PROTEIN BLAST of SEQ ID NO:8	
GI#	***DESCRIPTION***
4689086=AAD27752	p73 *[Barbus barbus]*
4530686=CAA03817	unnamed protein product [unidentified]
4803651=CAA72225	P73 splice variant *[Cercopithecus aethiops]*
4530690=CAA03819	unnamed protein product [unidentified]
4530684=CAA03816	unnamed protein product [unidentified]

TABLE 6 – HELIO p53

DNA BLAST of SEQ ID NO:9	
GI#	**DESCRIPTION**
N/A	No homologies found

PROTEIN BLAST of SEQ ID NO:10	
GI#	DESCRIPTION
2781308= 1YCSA	Chain A, p53-53bp2 Complex
1310770= 1TSRA	Chain A, p53 Core Domain In Complex With Dna
1310771= 1TSRB	Chain B, p53 Core Domain In Complex With Dna
1310772= 1TSRC	Chain C, p53 Core Domain In Complex With Dna
1310960= 1TUPA	Chain A, Tumor Suppressor p53 Complexed With Dna

BLAST analysis using each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous novel amino acids with respect to published sequences indicate the following: 7 amino acid residues for DMp53 and for

5   TRIB-Ap53, 6 amino acid residues for CPBp53, and 5 amino acid residues for TRIB-Bp53 and HELIOp53.

BLAST results for each of the p53 amino acid sequences to find the number of amino acid residues as the shortest stretch of contiguous amino acids for which there are no sequences contained within public database sharing 100% sequence similarity indicate the

10   following: 9 amino acid residues for DMp53, CPBp5, TRIB-Ap53, and TRIB-Bp53, and 6 amino acid residues for HELIOp53.

Example 9: *Drosophila* genetics

Fly culture and crosses were performed according to standard procedures at 22-25°C

15   (Ashburner, *supra*). Gl-DMp53 overexpression constructs were made by cloning a BclI HincII fragment spanning the DMp53 open reading frame into a vector (pExPress) containing glass multiple repeats upstream of a minimal heat shock promoter. The pExPress vector is an adapted version of the pGMR vector (Hay *et al.*, Development (1994) 120:2121-2129) which contains an alpha tubulin 3' UTR for increased protein stabilization

20   and a modified multiple cloning site. Standard P-element mediated germ line transformation was used to generate transgenic lines containing these constructs (Rubin and Spradling, *supra*). For X-irradiation experiments, third instar larvae in vials were exposed to 4,000 Rads of X-rays using a Faxitron X-ray cabinet system (Wheeling, IL).

25   ## Example 10: Whole-mount RNA *in situ* hybridization, TUNEL, and Immunocytochemistry

In situ hybridization was performed using standard methods (Tautz and Pfeifle, Chromosoma (1989) 98:81-85). DMp53 anti-sense RNA probe was generated by digesting DMp53 cDNA with EcoR1 and transcribing with T7 RNA polymerase. For

immunocytochemistry, third instar larval eye and wing discs were dissected in PBS, fixed in 2% formaldehyde for 30 minutes at room temperature, permeabilized in PBS+0.5% Triton for 15 minutes at room temperature, blocked in PBS+5% goat serum, and incubated with primary antibody for two hours at room temperature or overnight at 4°C. Anti-

5   phospho-histone staining used Anti-phospho-histone H3 Mitosis Marker (Upstate Biotechnology, Lake Placid, NY) at a 1:500 dilution. Anti-DMp53 monoclonal antibody staining used hybridoma supernatant diluted 1:2. Goat anti-mouse or anti-rabbit secondary antibodies conjugated to FITC or Texas Red (Jackson Immunoresearch, West Grove, PA) were used at a 1:200 dilution. Antibodies were diluted in PBS+5% goat serum. TUNEL

10   assay was performed by using the Apoptag Direct kit (Oncor, Gaithersburg, MD) per manufacturer's protocol with a 0.5% Triton/PBS permeabilization step. Discs were mounted in anti-fade reagent (Molecular Probes, Eugene, OR) and images were obtained on a Leica confocal microscope. BrDU staining was performed as described (de Nooij *et al.*, Cell. (1996)87(7):1237-1247) and images were obtained on an Axioplan microscope (Zeiss,

15   .Thornwood, NY).

Example 11: Generation of anti-DMp53 antibodies

        Anti-DMp53 rabbit polyclonal (Josman Labs, Napa, CA) and mouse monoclonal antibodies (Antibody Solutions Inc., Palo Alto, CA) were generated by standard methods

20   using a full-length DMp53 protein fused to glutathione-S-transferase (GST-DMp53) as antigen. Inclusion bodies of GST-DMp53 were purified by centrifugation using B-PER buffer (Pierce, Rockford, IL) and injected subcutaneously into rabbits and mice for immunization. The final boost for mouse monoclonal antibody production used intravenous injection of soluble GST-DMp53 produced by solubilization of GST-DMp53 in 6M GuHCl

25   and dialysis into phosphate buffer containing 1M NaCl. Hybridoma supernatants were screened by ELISA using a soluble 6XHIS-tagged DMp53 protein bound to Ni-NTA coated plates (Qiagen, Valencia, CA) and an anti-mouse IgG Fc-fragment specific secondary antibody.

30   ## Example 12: Functional analysis

        The goal of this series of experiments was to compare and contrast the functions of the insect p53s to those of the human p53. The DMp53 was chosen to carry out this set of experiments, although any of the other insect p53s could be used as well.

        p53 involvement in the cell death pathway

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To determine whether DMp53 can serve the same functions in vivo as human p53, DMp53 was ectopically expressed in *Drosophila* larval eye discs using *glass*-responsive enhancer elements. The *glass*-DMp53 (gl-DMp53) transgene expresses DMp53 in all cells posterior to the morphogenetic furrow. During eye development, the morphogenetic furrow

5   sweeps from the posterior to the anterior of the eye disc. Thus, gl-DMp53 larvae express DMp53 in a field of cells which expands from the posterior to the anterior of the eye disc during larval development.

Adult flies carrying the gl-DMp53 transgene were viable but had small, rough eyes with fused ommatidia (any of the numerous elements of the compound eye). TUNEL

10   staining of gl-DMp53 eye discs showed that this phenotype was due, at least in part, to widespread apoptosis in cells expressing DMp53. Results were confirmed by the detection of apoptotic cells with acridine orange and Nile Blue. TUNEL-positive cells appeared within 15-25 cell diameters of the furrow. Given that the furrow moves approximately 10 cell diameters per hour, this indicated that the cells became apoptotic 2-3 hours after

15   DMp53 was expressed. Surprisingly, co-expression of the baculovirus cell death inhibitor p35 did not block the cell death induced by DMp53 (Miller, J Cell Physiol (1997) 173(2):178-182; Ohtsubo *et al.*, Nippon Rinsho (1996) 54(7):1907-1911). However, DMp53-induced apoptosis and the rough-eye phenotype in gl-DMp53 flies could be suppressed by co-expression of the human cyclin-dependent-kinase inhibitor p21. Because

20   p21 overexpression blocks cells in the G1 phase of the cell cycle, this finding suggests that transit through the cell cycle sensitizes cells to DMp53-induced apoptosis. A similar effect of p21 overexpression on human p53-induced apoptosis has been described.

p53 involvement in the cell cycle

In addition to its ability to affect cell death pathways, mammalian p53 can induce

25   cell cycle arrest at the G1 and G2/M checkpoints. In the *Drosophila* eye disc, the second mitotic wave is a synchronous, final wave of cell division posterior to the morphogenetic furrow. This unique aspect of development provides a means to assay for similar effects of DMp53 on the cell. The transition of cells from G1 to S phase can be detected by BrdU incorporation. Eye discs dissected from wild-type third instar larvae displayed a tight band

30   of BrdU-staining cells corresponding to DNA replication in the cells of the second mitotic wave. This transition from G1 to S phase was unaffected by DMp53 overexpression from the gl-DMp53 transgene. In contrast, expression of human p21 or a *Drosophila* homologue, dacapo (de Nooij *et al.*, Cell (1996) 87(7):1237-1247; Lane *et al.*, Cell (1996) 87(7):1225-1235), under control of *glass*-responsive enhancer elements completely blocked DNA

replication in the second mitotic wave. In mammalian cells, p53 induces a cell cycle block in G1 through transcriptional activation of the p21 gene. These results suggest that this function is not conserved in DMp53.

In wild-type eye discs, the second mitotic wave typically forms a distinct band of cells that stain with an anti-phospho-histone antibody. In *gl*-DMp53 larval eye discs, this band of cells was significantly broader and more diffuse, suggesting that DMp53 alters the entry into and/or duration of M phase.

p53 response to DNA damage

The following experiments were performed to determine whether loss of DMp53 function affected apoptosis or cell cycle arrest in response to DNA damage.

In order to examine the phenotype of tissues deficient in DMp53 function, dominant-negative alleles of DMp53 were generated. These mutations are analogous to the R175H (R155H in DMp53) and H179N (H159N in DMp53) mutations in human p53. These mutations in human p53 act as dominant-negative alleles, presumably because they cannot bind DNA but retain a functional tetramerization domain. Co-expression of DMp53 R155H with wild-type DMp53 suppressed the rough eye phenotype that normally results from wild type DMp53 overexpression, confirming that this mutant acts as a dominant-negative allele *in vivo*. Unlike wild type DMp53, overexpression of DMp53 R155H or H159N using the *glass* enhancer did not produce a visible phenotype, although subtle alterations in the bristles of the eye were revealed by scanning electron microscopy.

In mammalian systems, p53-induced apoptosis plays a crucial role in preventing the propagation of damaged DNA. DNA damage also leads to apoptosis in *Drosophila*. To determine if this response requires the action of DMp53, dominant-negative DMp53 was expressed in the posterior compartment of the wing disc. Following X-irradiation, wing discs were dissected. TUNEL staining revealed apoptotic cells and anti-DMp53 antibody revealed the expression pattern of dominant-negative DMp53. Four hours after X-irradiation, wild type third instar larval wing discs showed widespread apoptosis. When the dominant-negative allele of DMp53 was expressed in the posterior compartment of the wing disc, apoptosis was blocked in the cells expressing DMp53. Thus, induction of apoptosis following X-irradiation requires the function of DMp53. This pro-apoptotic role for DMp53 appears to be limited to a specific response to cellular damage, because developmentally programmed cell death in the eye and other tissues is unaffected by expression of either dominant-negative DMp53 allele. The requirement for DMp53 in the

apoptotic response to X-irradiation suggests that DMp53 may be activated by DNA damage. In mammals, p53 is activated primarily by stabilization of p53 protein.

Although DMp53 function is required for X-ray induced apoptosis, it does not appear to be necessary for the cell cycle arrest induced by the same dose of irradiation. In the absence of irradiation, a random pattern of mitosis was observed in 3rd instar wing discs of *Drosophila*. Upon irradiation, a cell cycle block occured in wild-type discs as evidenced by a significant decrease in anti-phospho-histone staining. The cell cycle block was unaffected by expression of dominant-negative DMp53 in the posterior of the wing disc. Several time points after X-irradiation were examined and all gave similar results, suggesting that both the onset and maintenance of the X-ray induced cell cycle arrest is independent of DMp53.

p53 in normal development

Similar to p53 in mice, DMp53 does not appear to be required for development because widespread expression of dominant-negative DMp53 in *Drosophila* had no significant effects on appearance, viability, or fertility. Interestingly, *in situ* hybridization of developing embryos revealed widespread early embryonic expression that became restricted to primordial germ cells in later embryonic stages. This expression pattern may indicate a crucial role for DMp53 in protecting the germ line, similar to the proposed role of mammalian p53 in protection against teratogens.

Example 13: p53 RNAi experiments in cell culture

Stable *Drosophila* S2 cell lines expressing hemaglutinin epitope (HA) tagged p53, or vector control under the inducible metallothionen promoter were produced by transfection using pMT/V5-His (Invitrogen, Carlsbad, CA). Induction of DMp53 expression by addition of copper to the medium resulted in cell death via apoptosis. Apoptosis was measured by three different methods: a cell proliferation assay; FACS analysis of the cell population in which dead cells were detected by their contracted nuclei; and a DNA ladder assay. The ability to use RNAi in S2 cell lines allowed p53 regulation and function to be explored using this inducible cell-based p53 expression system.

Preparation of the dsRNA template: PCR primers containing an upstream T7 RNA polymerase binding site and downstream DMp53 gene sequences were designed such that sequences extending from nucleotides 128 to 1138 of the DMp53 cDNA sequence (SEQ ID NO:1) could be amplified in a manner that would allow the generation of a

DMp53-derived dsRNA. PCR reactions were performed using EXPAND High Fidelity (Boehringer Mannheim, Indianapolis, IN) and the products were then purified.

DMp53 RNA was generated from the PCR template using the Promega Large Scale RNA Production System (Madison, WI) following manufacturer's protocols. Ethanol precipitation of RNA was performed and the RNA was annealed by a first incubation at 68°C for 10 min, followed by a second incubation at 37°C for 30 min. The resulting dsRNA was stored at -80°C.

RNAi experiment in tissue culture: RNAi was performed essentially as described previously (http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html). On day 1, cultures of Drosophila S2 cells were obtained that expressed pMT-HA-DMp53 expression plasmid and either 15 µg of DMp53 dsRNA or no RNA was added to the medium. On the second day, $CuSO_4$ was added to final concentrations of either 0, 7, 70 or 700 µM to all cultures. On the fourth day, an alamarBlue (Alamar Biosciences Inc., Sacramento, CA) staining assay was performed to measure the number of live cells in each culture, by measuring fluorescence at 590 nm.

At 7µM $CuSO_4$, there was no change in cell number from 0 µM $CuSO_4$ for RNAi treated or untreated cells. At 70 µM $CuSO_4$, there was no change in cell number from 0 µM $CuSO_4$ for the RNAi-treated category. However, the number of cells that were not treated with RNAi dropped by 30%. At 700 µM $CuSO_4$, the number of cells that were treated with RNAi dropped by 30% (as compared with 0µM $CuSO_4$), while the number of cells that were not treated with RNAi dropped by 70%.

These experiments showed that p53 dsRNA rescued at least 70% of the cells in the p53 inducible category, since some cell loss might be attributable to copper toxicity. Results of these experiments demonstrate that DMp53 dsRNA rescues cells from apoptosis caused by inducing DMp53 overexpression. Thus, this experimental cell-based system represents a defined and unique way to study the mechanisms of p53 function and regulation.